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(57) Abstract

The present invention provides DNA constructs comprising a promoter DNA sequence and a DNA sequence coding for human serum albumin. In one embodiment the human serum albumin sequence comprises at least one, but not all, of the introns in the naturally occurring gene encoding for the HSA protein. In another embodiment the DNA constructs comprise a 5′ regulatory sequence which directs the expression and secretion of HSA protein in the milk of a transgenic animal. Preferably, the promoter gene is a milk protein promoter sequence such as β-lactoglobulin. The present invention also provides transgenic animals which secrete HSA in the milk of lactating females. The present invention also provides vectors comprising the constructs of the present invention.

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TRANSGENIC PROTEIN PRODUCTION

Field of the Invention

This invention relates to HSA-encoding DNA molecules, vectors containing same and HSA-producing transgenic mammals.

Background of the Invention

Human serum albumin (HSA) is a globular, non-glycosylated protein (MW 65,000) synthesized by the liver. Circulating in the blood stream at levels of 42 grams/liter, it is the most abundant serum protein. HSA is involved in a number of essential functions, including sustaining normal bloodstream osmolarity, regulating blood pressure and transporting fatty acids, amino acids, bile pigments and numerous small molecules. Clinically, HSA is used in large quantities to replace blood volume in acute phase conditions such as trauma and severe burns or surgical procedures. Currently, medical supply of HSA depends on the fractionation of donated human blood. At the present time, the cost of purifying HSA from blood is relatively low, since HSA as well as other blood products can be simultaneously purified from the same source. However, as other blood products, such as coagulation factors, are produced by biotechnology instead of purified from human blood, market dynamics will increase the relative cost of purification of HSA from blood. The threat of a diminishing supply of donated blood, rising costs of purifying HSA from blood and the potential risk of contamination with infectious viruses that cause hepatitis, AIDS and other diseases make an alternative source of production of large quantities of HSA desirable. As such, alternative approaches to the production of large quantities of HSA are required.

Recombinant DNA technology has been used increasingly over the past decade for the production of commercially important biological materials. To this end, the DNA sequences encoding a variety of medically important human

proteins have been cloned. These include insulin, plasminogen activator, alpha-antitrypsin and coagulation factors VIII and IX.

The expression of DNA sequences encoding these and other proteins has been suggested as the ideal source for the production of large quantities of mammalian proteins. A variety of hosts have been utilized for the production of medically important proteins including bacteria, yeast, cultured cells and animals. In practice, bacteria and yeast often prove unsatisfactory as hosts because the foreign proteins are often unstable and are not processed correctly. In bacteria, HSA is produced as an insoluble aggregate which requires processing to yield the mature, soluble protein. HSA has also been produced in the yeast Saccharomyces, but at low levels and with a high proportion being either fragmented, cell associated or insoluble (Sleep et al.., 1990, Bio/Technology 8:42-46; Etcheverry et al.., 1986, Bio/Technology 4:729-730; Quirk et al.., 1989, Biotech. Appl Biochem. 11:273-287).

In light of this problem, the expression of cloned genes in mammalian tissue culture has been attempted and has, in some instances, proved a viable strategy. However, batch fermentation of animal cells is an expensive and technically demanding process. Transgenic animals have also been proposed as a source for the production of protein products. The production of transgenic livestock offers a number of potential applications including "Molecular Farming" (also referred to as Genetic Farming) where proteins of medical or commercial importance are targeted for high level expression and production in the mammary gland with subsequent secretion into the milk of such genetically engineered animals. The feasibility of this approach was first tested in transgenic mice.

WO-A-8800239 discloses transgenic animals which secrete a valuable pharmaceutical protein, in this case Factor IX, into the milk of transgenic sheep. EP-A-0264166 also discloses the general idea of transgenic animals secreting pharmaceutical proteins into their milk.

Early work with transgenic animals, as represented by WO-A-8800239, has used genetic constructs based on cDNA coding for the protein of interest. The cDNA will be smaller than the natural gene, assuming that the natural gene has introns, and for that reason is easier to manipulate. It is desirable for

commercial purposes to improve upon the yields of proteins produced in the milk of the transgenic animal.

Brinster et al. (PNAS 85 836-840 (1988)) have demonstrated that the transcriptional efficiency of transgenes having introns in transgenic mice is increased over that of cDNA. Brinster et al.. show that all the exons and introns of a natural gene are important both for efficient and for reliable expression (that is to say, both the levels of the expression and the proportion of expressing animals) and is due to the presence of the natural introns in that gene. It is known that in some cases this is not attributable to the presence of tissue-specific regulatory sequences in introns, because the phenomenon is observed when the expression of a gene is redirected by a heterologous promoter to a tissue in which it is not normally expressed. Brinster et al.. suggested that the effect is peculiar to transgenic animals and is not seen in cell lines. However, Huang and Gorman (1990, Nucleic Acids Research 18:937-947) have demonstrated that a heterologous intron linked to a reporter gene can increase the level of expression of that gene in tissue culture cells.

The problems of yield and reliability of expression can not be overcome by merely following the teaching of Brinster et al.. and inserting into mammalian genomes transgenes based on natural foreign genes as opposed to foreign cDNA. First, as mentioned above, natural genes having introns are larger than the cDNA coding for the product of the gene since the introns are removed from the primary transcription product before export from the nucleus as mRNA. It is technically difficult to handle large genomic DNA.

Secondly, the longer the length of manipulated DNA, the greater chance that restriction sites occur more than once, thereby making manipulation more difficult. This is especially so given the fact that in most transgenic techniques, the DNA to be inserted into the mammalian genome will often be isolated from prokaryotic vector sequences (because the DNA will have been manipulated in a prokaryotic vector, for choice). The prokaryotic vector sequences usually have to be removed, because they tend to inhibit expression. So the longer the piece of DNA, the more difficult it is to find a restriction enzyme which will not cleave it internally.

Attempts to achieve protein expression utilizing cDNA encoding the protein instead of the full length gene, have generally resulted in low protein yields. A number of workers recognized the desirability of improving upon the yields and reliability of transgenic techniques obtained when using constructs based on cDNA.

Archibald et al.. (WO90/05188) noted that with certain proteins higher yields (than could be obtained utilizing cDNA) could be obtained when at least some of the naturally occurring introns were utilized. Palmiter et al.. (1991, Proc. Natl. Acad. Sci, USA 88:478-482) also found that the level of expression of a transgene was higher when the transgene included some introns as compared with the transgene composed of a cDNA. However, the level of expression with less then all of its natural introns was reduced when compared to the level of expression obtained with the entire gene with all of its introns.

Summary of the Invention

The present invention provides DNA constructs comprising a promoter DNA sequence and a DNA sequence coding for human serum albumin. In one embodiment the human serum albumin sequence comprises at least one, but not all, of the introns in the naturally occurring gene encoding for the HSA protein. In another embodiment the DNA constructs comprise a 5' regulatory sequence which directs the expression and secretion of HSA protein in the milk of a transgenic animal. Preferably, the promoter gene is a milk protein promoter sequence such as ß-lactoglobulin, whey acidic protein or ß-casein. Most preferably the secreted protein is human serum albumin. The present invention also provides vectors comprising the constructs of the present invention.

The DNA construct of the present invention encoding HSA comprises two contiguous exons encoding HSA and an HSA intron. In a preferred embodiment, the DNA construct of the present invention provides for expression of HSA in mammalian cells and milk at higher levels than the naturally occurring HSA gene or HSA cDNA. In a most preferred embodiment the DNA construct of the present invention comprises HSA exons and introns selected from the group consisting of introns 1-6, 7-14, 1+7-14, 1 + 2 +12-14, 2 +12-14, 2 + 7-14 and 1+ 2 + 7-14.

In another embodiment , a DNA construct of the present invention encoding HSA comprises one but not all of the first 7 introns of the HSA gene, and one of the last 7 introns of the HSA gene.

In another embodiment, a DNA construct comprising DNA sequences encoding human serum albumin under the control of a mammary tissue specific promoter, said DNA construct expressed by the mammary glands of a lactating female transgenic mammal is provided.

The present invention also provides a transgenic mammal having incorporated into its genome a DNA construct comprising DNA sequences encoding human serum albumin operably linked to a mammary tissue specific promoter, said DNA construct expressed by the mammary glands of a lactating female transgenic mammal. Preferably, the promoter is the ß-lactoglobulin protein promoter.

The present invention also provides a method of making a transgenic mammal having incorporated into its genome a DNA construct encoding human serum albumin and a mammary tissue specific promoter, said DNA construct expressed by mammary glands of a lactating female transgenic mammal comprising providing a DNA construct containing the ß-lactoglobulin promoter operably linked with nucleotide sequence encoding human serum albumin.

The present invention also provides a transgenic mammal which secretes HSA in the milk of lactating females.

Other and further objects features and advantages will be apparent from the following description of the presently preferred embodiments of the invention, given for the purposes of disclosure when taken in conjunction with the accompanying drawings.

Brief Description of the Figures

The invention will be more readily understood from a reading of the following specification and by reference to the accompanying drawings forming a part thereof:

Figure 1 is a map of the human serum albumin gene (1A) and the sequence of the HSA gene (1B).

Figure 2 is a graphical depiction of the DNA constructs.

Figure 3 demonstrates a fluorograph of SDS PAGE from in vitro expression analysis.

Figure 4 demonstrates a Southern blot analysis of transgenic mice carrying HSA constructs.

Figure 5 demonstrates a dot blot analysis (5A) and a Western blot analysis (5B) of BLG expression in the milk of transgenic animals.

Figure 6 demonstrates a dot blot analysis for detection (6A) and quantitation (6B) and a Western analysis (6C) of HSA expression in the milk of transgenic animals.

Figure 7 demonstrates a non-denaturing gel analysis of protein in the milk of transgenic animals by Coomassie stain (7A) and Western blot analysis (7B) of HSA expression in the milk of transgenic animals.

Figure 8 demonstrates an HSA RNA analysis (Northern) of tissues of transgenic animals.

Figure 9 demonstrates in situ detection of HSA RNA.

Figure 10 demonstrates a Western analysis of HSA expression by mammary explants of transgenic animals.

Figure 11 demonstrates the immunohistochemical detection of HSA and ß-casein in mammary glands of virgin and lactating transgenic mice of strain #23.

Figure 12 demonstrates the immunohistochemical detection of HSA and β-casein in mammary glands of virgin and lactating transgenic mice of strain #69

Figure 13 demonstrates the immunohichemical detection of HSA β-casein in mammary glands of virgin and lactating control non-transgenic mice.

Figure 14 demonstrates a Northern analysis of HSA, BLG and β-casein RNA expressed in the mammary glands of virgin, pregnant and lactating transgenic and non-transgenic control mice.

Figure 15 demonstrates (by metabolic labeling and immunoprecipitation) the synthesis and secretion of HSA and BLG from mammary explants of virgin, pregnant and lactating transgenic mice.

Figure 16 demonstrates (by metabolic labeling and immunoprecipitation) the hormonal control of HSA and BLG synthesis and secretion from mammary explants, cultured over time, from virgin transgenics.

Figure 17 demonstrates Western immunoanalysis of HSA secreted in milk of several transgenic mouse strains.

Figure 18 demonstrates (by metabolic labeling and immunoprecipitation) expression and secretion of HSA from mammary explants as evaluated under several conditions.

Figure 19 demonstrates a correlation between the levels of expression of HSA in the milk of transgenic strains and the levels of secretion of HSA from mammary explants from virgin females of these same strains.

Figure 20 demonstrates a Western immunoanalysis of HSA secretion (upper panel) and BLG secretion, by Coomassie staining, (lower panel) by mammary explants of aborted transgenic goats.

Detailed Description of the Preferred Embodiments

Definitions:

The term "naturally occurring HSA gene" means the DNA sequences which encode the HSA protein and includes exons and introns in their native positional relationships. The naturally occurring HSA gene has been sequenced and the sequence reported by Minghetti et al., J. Biol. Chem. 261:6747-6757 (1986). As used herein, HSA base pair (bp) positions are related to this published sequence which is also shown on Figure 1B. The numbering system used herein is defined such that the first bp (A) of the HSA translational initiation codon (ATG) is numbered as bp 1776 which is bp 40 on the sequence shown on Figure 1B. In the native state the HSA gene includes 5' flanking sequences (including promoter sequences) which are responsible for initiation and regulation of transcription and expression and 3' flanking sequences, as used herein the term "naturally occurring HSA gene" need not include these flanking sequences. In the constructs of the present invention the native flanking sequences may be absent or substituted by a heterologous sequence.

As used herein, the term "intron" (also called intervening sequences) are those sequences of a naturally occurring gene which are included within the transcription unit of the gene but do not encode the natural gene product protein. The introns are transcribed into the precursor RNA, but are removed during the processing (splicing) of the RNA to its mature form, messenger RNA (mRNA). The introns are located between flanking exons. In this specification the term "intron" includes the whole of any natural intron or part thereof.

As used herein the term "exon" refers to DNA sequences which are included within the transcription unit of the gene and maintained in the mature mRNA following processing and which encode the gene product protein. When an intron is deleted or removed from the naturally occurring gene, the two exons which naturally flank that intron become adjoined as contiguous exons.

The DNA constructs of the present invention will generally be suitable for use in expressing the HSA protein in mammalian cells and, preferably, in the mammary gland of a transgenic animal with subsequent secretion of HSA in the milk. The DNA constructs of the present invention comprise DNA sequences encoding the HSA protein together with 5' flanking regulatory elements which include promoter sequences. When expression in mammary tissue is desired the 5' regulatory sequences are chosen which directs the expression and secretion of HSA protein in the milk of a transgenic animal. Preferably, the promoter is a milk protein promoter sequence such as ß-lactoglobulin, whey acidic protein or ß-casein. When expression of the HSA encoding construct of the present invention in tissue culture cells is desired, an enhancer sequence may be included in the construct. Enhancer elements may be derived from SV40, human cytomegalovirus or any other source. The choice of enhancer will be known to one of skill in the art. The constructs of the present invention also comprise polyadenylation (poly A) signals and sites. The polyadenylation signal may be a homologous signal encoded by the native HSA gene or may be heterologous, for example, the BLG or SV40 poly A sites. The choice of promoter, poly A or other regulatory elements will be known to those of skill in the art.

The species of animals selected for expression is not particularly critical, and will be selected by those skilled in the art to be suitable for their needs.

Clearly, if secretion in the mammary gland is the primary goal, as is the case with preferred embodiments of the invention, it is essential to use mammals. Suitable laboratory mammals for experimental ease of manipulation include mice and rats. Domestic farm animals such as rabbits, cows, pigs, goats and sheep provide larger yields than other mammals. Preferably, sheep and goats are utilized because of the relative annual milk production in relation to generation time, experimental production time, and cost.

According to another aspect of the invention, there is provided a vector (or DNA construct) comprising a genetic construct comprising at least one HSA intron and fewer than all of the HSA introns which vector when used to transfect a mammalian cell expresses HSA at a higher level of expression than the full naturally occurring HSA gene.

According to another aspect of the invention, there is provided a mammalian or other animal cell comprising a construct as described above. According to a sixth aspect of the invention, there is provided a transgenic mammal or other animal comprising a genetic construct as described above integrated into its genome. It is particularly preferred that the transgenic animal transmits the construct to its progeny, thereby enabling the production of at least one subsequent generation of producer animals.

The DNA sequence of the naturally occurring HSA gene has been determined. Figure 1 demonstrates a map of the HSA gene and its sequence..

In order to make the DNA constructs of the present invention several different approaches were required. The ovine BLG gene was cloned from high molecular weight liver DNA as two EcoR I subgenomic fragments (5'-half approximately 4.3 kb and 3'-half approximately 4.4 kb) into the EcoR I site of lambda gt10 vector. The two halves were subcloned into pGEM-I, joined together at their EcoR I sites within the transcriptional unit, by using adaptor oligonucleotides which destroyed the EcoR I sites at the gene's 5'- and 3'-ends and which introduced Sal I sites at these positions. A unique SnaB I site was introduced into the Pvu II site within the 5'-untranslated region of exon 1. This results in vector p585 for the expression of β-lactoglobulin and contains approximately 3kb of 5'-flanking promoter sequences. Constructs are represented in Figs. 2 A and 2B. The BLG 5'-flanking sequences were

extended by cloning from ovine liver DNA a Hind III subgenomic fragment extending from the Hind III site within the transcriptional unit upstream approximately 8 kb to a 5'-Hind III site (p644 and p643) or a Sac I subgenomic fragment extending from the Sac I site within the original 5'-flanking sequences upstream approximately 8.6 kb to another Sac I site (p646 and p647). The former constructs include approximately 5.5 kb of 5'-flanking promoter sequences. The latter construct includes approximately 10.8 kb of these sequences.

An HSA cDNA was isolated from a lambda gt11-human liver cDNA library. The cDNA contains the complete HSA coding sequence including the prepropeptide sequences as well as 20 bp of 5'-untranslated and 141 bp of 3'-untranslated sequence. The HSA cDNA was inserted into the Pvu II site within the untranslated region of BLG exon 1 in the same orientation as the BLG coding sequence resulting in vector p575. A DNA fragment encompassing the HSA genomic sequence including part of exon 1, intron 1, exon 2, intron 2 and part of exon 3 was produced by PCR using a 5'-oligonucleotide primer which overlaps the native BstEII site in HSA exon 1 and a 3'-oligonucleotide primer which overlaps the native Pvu II site in HSA exon 3 using high molecular weight DNA purified from human lymphocytes as a template. The cDNA region between the BstEII site in exon 1 encoding region and the Pvu II site in exon 3 encoding region was replaced with the corresponding genomic fragment (2401 bp) from the PCR product. This resulted in an HSA minigene possessing introns 1 and 2 in their native locations, as included in vector p607. In order to introduce the first intron of the HSA gene into the HSA cDNA we first introduced a Cla I site into the region of the HSA cDNA which is derived from HSA exon 2 by replacing a G with an A in the third base position of the codon for the 34th amino acid of the HSA protein including the prepropeptide, by in vitro mutagenesis. The altered codon encodes arginine as did the original. HSA intron 1 DNA with flanking exon sequences was generated by PCR using a 5'-oligonucleotide primer which overlaps the native BstEII site in exon 1 and a 3'-oligonucleotide primer which overlaps, and contains, the Cla I site introduced into exon 2 encoding DNA. A clone of the genomic PCR product containing exon 1 through exon 3 sequences was used as template for PCR generation of intron 1 and flanking sequences. The cDNA region between the native BstEII site in exon 1 encoding region and the introduced Cla I site in exon 2 encoding region was replaced with the corresponding genomic

fragment from the PCR product. The resulting HSA minigene possesses intron 1 in its native location as included in vectors p599, p600, p598, p643 and p647.

The deletion of the BLG coding sequence was accomplished by deleting sequences between the introduced SnaB I site within the 5'-untranslated region of BLG exon 1 and the native Xma I site within the 3'-untranslated region of BLG exon 7 prior to introduction of HSA sequences, as seen in vectors p600 and p607. This vector maintains most of the untranslated BLG exon 7 including its polyadenylation signal and site as well as sequences 3' of the BLG transcription unit. The SV40 early gene (T and t) polyadenylation signal and site downstream of HSA sequences in vectors p598, p643 and p647 as well as other vectors was obtained from SV40 DNA by restriction with BclI at its 5'-end (SV40 map position 2770) and BamH I at its 3'-end (SV40 map position 2533). In these vectors all BLG sequences downstream of the introduced SnaB I site in the 5'-untranslated BLG exon 1 including coding sequence, polyadenylation signal and site and 3'-flanking sequences were deleted.

In order to obtain HSA introns 3-14, the HSA gene was cloned from human placental DNA. Three Nco I sites within the HSA gene sequence were identified. The first Nco I site lies about 275 base pairs upstream of HSA exon 1. The second site lies within exon 7 and the third site lies about 227 base pairs downstream of exon 15. Digestion of human high molecular DNA with Nco I released two fragments of 8079 and 9374 base pairs which together encompass the entire HSA gene. The 8079 base pair fragment represents the 5'-half of the HSA gene while the 9374 base pair fragment represents the 3'-half of the gene. These fragments were used to make 2 separate subgenomic DNA libraries. HSA clones from these libraries were identified using an HSA cDNA probe. A clone containing the 5'- half of the HSA gene to exon 7 was designated p650. A clone (p651) identified as containing sequences of the 3'-half of the HSA gene were found to have an internal deletion of HSA sequences. Clone p651, did, however, contain HSA sequences extending from the Asp I site within HSA exon 12 through the Nco I site downstream of HSA exon 15.

In order to clone the HSA gene sequences between exon 7 and exon 12 so as to obtain that region which includes introns 7-11, PCR technology was utilized. Four PCR reactions were set up using synthetic priming oligonucleotides homologous to desired regions of the HSA gene containing useful restriction sites. The PCR reactions were designed so that the upstream end of reaction #1 overlapped the Nco I site within exon 7. The downstream end of reaction #1 overlapped the Avr II site within intron 8. This same Avr II site was overlapped by the upstream end of PCR reaction #2. The downstream end of PCR reaction #2 overlapped the Hind III site within intron 8. This Hind III site was also overlapped by the upstream end of PCR reaction #3. The downstream end of PCR reaction #3 overlapped the XhoI site in intron 10 which was also overlapped by the upstream end of PCR reaction #4. The downstream end of PCR reaction #4 overlapped the Asp I site in exon 12. By this PCR strategy the entire region desired was obtained and adjacent PCR products were joined together using overlapped restriction sites. The products of PCR #1 and #2 reactions were ligated together at their common Avr II site within HSA intron 8 and cloned into plasmid pGEM-1 resulting in construct p679. This construct contains HSA sequences extending from the Nco I site in exon 7 to the Hind III site in the downstream end of intron 8. The product of PCR #3 and #4 reactions were ligated together at their common XhoI site within HSA intron 10 and cloned into plasmid pGEM-2 resulting in construct p676. This construct contains HSA sequences extending from the same Hind III site in the downstream end of intron 8 to the Asp I site in exon 12.

When taken together with construct p650, containing the HSA gene sequences from the Nco I site upstream of HSA exon 1 (HSA gene base pair position 1462) to the Nco I site in exon 7 (HSA gene base pair position 9541) and construct p651, containing HSA gene sequences extending from upstream of the Asp I site in exon 12 (HSA gene base pair position 15592) to the Nco I site downstream of exon 15 (HSA gene base pair position 18915) these constructs, p679 and p676, complete the cloning of the entire HSA gene.

In order to assess the contribution of introns to the level of expression of HSA in the milk of transgenic animals, constructs comprising the HSA exons and various combinations of introns were constructed. Details of the various constructions are given in the examples which follow.

The constructs of the present invention were tested for their ability to support expression of HSA protein in vitro in tissue culture cells and in vivo in transgenic animals. The in vitro expression of HSA by the constructs of the present invention is described in detail in Example 15. The natural in vivo regulation of expression of milk proteins under the control of the native promoters (e.g. BLG) is complex and requires the influence of hormones and specific cell-cell interactions. The BLG promoter is not usually active in tissue culture cells. In order to drive expression in the COS-7 cells chosen for these in vitro tests, an SV40 enhancer was introduced within the BLG promoter. Details of the construction of the constructs of the present invention having the SV40 enhancer are given in Example 15. A transient assay for HSA expression in COS-7 cells was used to test the constructs. Briefly, constructs of the present invention were transfected into COS-7 cells, incubated for 48-72 hours. Expression of HSA was determined by metabolically labeling de novo synthesized proteins with ^{35}S-methionine and immunoprecipitating labeled HSA protein, which had been secreted into the media supernatants, with HSA specific antibodies. Precipitated HSA was analyzed by SDS-PAGE and HSA bands detected by fluorography.

COS-7 cells transfected with a construct which contained HSA cDNA, lacking introns, (p658) expressed HSA at low levels. Expression of HSA protein was also relatively low even when some of the introns were included in the construct. However, selection of certain combinations of introns [p656 (introns 1-6), p684 (introns 7-14), p695 (introns 2+12-14), p697 (introns 1+2+12-14), p693 (introns 1+7-14), p692 (introns 2+7-14), and p698 (introns 1+2+7-14)] supported expression of HSA protein at levels equal to or even greater than the full length HSA gene [p685 (introns 1-14; full length)]. In vitro analyses are shown in Figs. 3A and 3B and summarized in Table 1.

Table 1

In Vitro Construct	HSA Introns Included	Level of In Vitro Expression	Homologous In Vivo Construct	In Vivo Expression in Transgenic Milk
p615	0 (cDNA) (BLG coding seq.) (BLG 3'-seq.)	1	p575 (BLG coding seq.) (BLG 3'-seq.)	0 / 8
p608	1	2	p598	See below
p606	1 (BLG 3'-seq.)	2	p600 (BLG 3'-seq.)	0 / 6
			p599 (BLG coding seq.) (BLG 3'-seq.)	0 / 5
p610	1 & 2 (BLG 3'-seq.)	3	p607 (BLG 3'-seq.)	4 / 6 (1-35 µg/ml)
* p658	0 (cDNA)	1		
* p659	1	2	p598	1 / 5 (2.5 mg/ml)
			p643 (5.5 kb BLG 5'-seq.)	0 / 2
			p 647 (10.8 kb BLG 5'-seq.)	1 / 6 (2 µg/ml)
* p691	2	3		
* p660	1 & 2	3	p607 (BLG 3'-seq.)	see above
* p656	1 - 6	6	p652	4/12 (0.002 - 10 mg/ml)
			p654 (5.5 kb BLG 5'-seq.)	0/5
* p682	12 - 14	2	p688	In progress
* p684	7 - 14	6	p687	3/7 (0.005-5 mg/ml)
* p685	1 - 14	6	p686	1/2 (1 µg/ml)
* p694	1 + 12 - 14	4		
* p695	2 + 12 - 14	5		
* p697	1 + 2 + 12 - 14	7	p812	1 (0.8-1 mg/ml) 3 not yet determ.
* p693	1 + 7 - 14	6		
* p692	2 + 7 -14	8	p696	3/4 (0.002-2.5 mg/ml)
* p698	1 + 2 + 7 - 14	8		

In vitro constructs marked with (*) vary only in presence of HSA introns. (BLG 5'-sequences, SV40 enhancer and 3'-SV40 poly A are the same)

Except where indicated constructs lack the BLG coding sequences and BLG 3'-sequences including poly A. Unless indicated the SV40 poly A site was utilized. Unless indicated the 3kb BLG 5'-flanking sequences (promoter) were utilized.

In vitro expression level ranges 1 (low expression) to 8 (high expression) are semiquantitative comparisons where each increment represents several fold to many fold differences in level of expression.

"Not yet determ." means that transgenic produced but presence of HSA in milk not yet determined. "Not yet quant." means that expression of HSA in the milk of the transgenic has been shown but the amount not yet quantitated.

Expression of HSA in tissue culture cells from these constructs demonstrated that the level of expression of HSA is modulated by the specific complement of HSA introns, i.e., the number of HSA introns present in the construct, the specific introns incorporated, the relative locations of introns, and the synergism between specific introns. Several fold higher levels of expression are obtained with constructs containing HSA minigenes with specific subsets of introns as compared with the entire HSA gene with all of its introns. Levels of expression of HSA are particularly high when supported by HSA minigenes which comprise one but not all of the first 7 introns of the HSA gene and one of the last 7 introns of the HSA gene. There are 5 Alu sequences (family of repeated DNA sequences) within HSA introns. Three of these Alu sequences are located within the first 7 introns and 2 are located within the last 7 introns. Intron 2 has 2 Alu sequences, introns 7, 8, and 11 each have 1 Alu sequence. There appears to be an association between the presence of the Alu sequences within introns and the introns' positive effect on resultant levels of expression obtained with HSA minigenes.

In vivo expression of heterologous protein in transgenic animals by the constructs of the present invention was assessed by injecting the constructs of the present invention into murine oocytes to produce transgenic mice. Transgenic mice were produced following the general methods described by Hogan et al.., "Manipulating the mouse embryo: a laboratory manual" CSHL (1986). The details are further described in Example 16. Two different heterologous proteins, BLG and HSA, were expressed in the milk of the transgenic mice. Mice carrying the BLG or the BLG/HSA constructs of the present invention were detected by analysis of somatic DNA from the tails of newborn mice utilizing a ^{32}P DNA probe which recognizes both BLG and HSA

DNA sequences. Details of the assay are described in Example 16. In contrast to the in vitro assay, no enhancer was needed to drive the expression in transgenics of proteins under the direction of the BLG promoter. Female mice that were determined to carry the construct of the present invention, when mature were mated to non-transgenic males. Milk was collected from nursing transgenic females after parturition and the milk analyzed for the presence and amount of heterologous protein (BLG or HSA). Founder male transgenic mice were bred to non-transgenic females to produce female offspring to test for the expression of heterologous proteins in their milk. Heterologous protein expressed in milk was detected by a dot blot assay using antibodies to BLG or HSA protein. BLG expression in milk was detected by spotting the milk sample onto a nitro- cellulose filter. Anti-BLG antibodies were then contacted with the filter. ^{125}I-Protein A was then contacted with the filter to bind quantitatively to the bound antibodies. HSA expression in milk was detected by spotting the milk sample onto a nitrocellulose filter. Iodinated anti-HSA monoclonal antibodies were then contacted with the filter. The radioactivity was determined by autoradiography and correlated with standards by densitometry of the autoradiographs to quantitate the amount of heterologous protein expressed in the transgenic milk.

The capability of the BLG promoter to promote expression of BLG and HSA was tested both in vitro and in vivo. A 3kb DNA fragment upstream of the BLG transcription unit (i.e. the 5'-flanking region) was utilized in most of the constructs of the present invention. The efficacy of this 3kb promoter was tested by making transgenic mice utilizing construct p585 which contain the BLG gene under the control of the 3kb BLG promoter.

All transgenic mouse strains produced, carrying the native sheep BLG gene (construct p585), expressed BLG at high levels in the mammary gland and milk as determined by dot blot. An example is shown in Fig. 4. Levels ranged from 1 to 8.5 mg/ml BLG (see Table 2). This is somewhat lower than the range (3-23 mg/ml) found by Simons et al.. (1987, Nature 328:530-532) utilizing 4.3 kb BLG 5'-flanking sequences. However, no increase in BLG expression was observed with 5'-flanking sequences of 5.5 kb (construct p644) or 10.8 kb (p646). These results demonstrate that the BLG 3kb 5'-flanking sequences in the constructs of the present invention contain all the 5'-control elements necessary to direct high level expression to the mammary gland.

This 3kb BLG promoter fragment was also used in constructs comprising all or parts of the HSA DNA. This 3kb fragment proved sufficient to promote BLG and HSA expression both in vitro and in vivo.

The milks of lactating females from transgenic lines produced from BLG/HSA constructs were analyzed for the presence of HSA by immuno-dot blot using iodinated anti-HSA monoclonal antibodies. An example is shown in Fig. 5A. Our initial attempt to produce transgenic mice expressing HSA in their milk was by introducing the HSA cDNA into the 5'-untranslated region of the first exon of the BLG gene of vector p585, resulting in vector p575. None of the 8 lines secreted detectable levels of the human protein (Table 2). It appeared that although our BLG vector was able to drive expression of its own BLG gene, it was unable to support the expression of the inserted HSA cDNA. Others have found that in transgenics, the levels of expression of heterologous genes under the control of a variety of 5'-regulatory elements (promoters) were increased by the incorporation of introns into the heterologous transcripts. Therefore, we tested a series of vectors in which the sheep BLG promoter was fused to HSA minigenes containing a variety of intron combinations.

Analysis of expression from one HSA minigene, containing HSA intron 1, within 3 constructs (p599, p600, p598) demonstrated only 1 transgenic line (#23) out of 16 produced expressing detectable levels of HSA in its milk. Inclusion of HSA intron 1 alone in the constructs of the present invention is not sufficient to obtain a high percentage of transgenic lines which express. As shown on Fig. 5B, female mice of line 23 secrete high levels, greater than 2 mg/ml of HSA into their milk, and have stably transmitted this ability to their progeny for over two years.

Mouse milk contains a significant amount of endogenous mouse serum albumin which co-migrates with human serum albumin in SDS-PAGE gels (data not shown). Immuno-detection assays demonstrated that the anti-HSA monoclonal antibody specifically detected the human protein and not the mouse protein. The human and mouse proteins were also distinguishable by their distinct electrophoretic mobilities on native polyacrylamide gels. Milk from expressing line 23 clearly contains both the human (low mobility) and mouse (high mobility) albumins as seen by generalized protein staining with

coomassie (Fig. 6). The lower mobility band was confirmed to be HSA by native gel and immunoblot analysis (Fig. 6).

The secreted HSA protein behaves in a manner indistinguishable from purified HSA or the HSA found in human milk in its electrophoretic mobility through native gels as well as in denaturing gels. In native gels the human protein migrates with a different mobility from endogenous mouse serum albumin.

Reproducible expression of HSA in the milk of transgenics was achieved first only when the first 2 HSA introns were included in the construct (p607) with 4 of the 6 transgenic lines examined expressing HSA in their milk. Although an improvement in the frequency of expressing transgenics (penetrance), the levels of expression were disappointingly low (1-35 µg/ml).

A significant improvement in both the number of transgenics which express HSA in their milk (penetrance) as well as the levels of expression resulted from the inclusion of additional HSA introns or intron combinations within transgenic vectors. Four of twelve transgenic strains generated from vector p652 (HSA introns 1-6) express HSA in their milk with two strains expressing HSA above 1 mg/ml (one of which expresses at about 10 mg/ml). Three of seven strains generated from p687 (HSA introns 7-14) expressed HSA with one strain expressing at about 4-5 mg/ml. Three of four strains generated from p696 (HSA introns 2 + 7-14) expressed HSA with one strain expressing at about 2.5 mg/ml. Four transgenic strains have thus far been generated from vector p812 (HSA introns 1 + 2 + 12-14). Only one of these strains is old enough to have already been tested for the expression of HSA in milk and, in fact, this one strain does express high levels of HSA at about 0.8-1.0 mg/ml. Only two strains have been generated from p686 (HSA gene; i.e.. all HSA introns 1-14). One of the two expressed HSA but at low levels.

The overall in vivo results correlate with the in vitro expression results. That is, extremely low levels of HSA are expressed from the cDNA construct in vitro and no HSA is detectably expressed from the cDNA construct in vivo in transgenics. The inclusion of intron 1 into the in vitro construct results in slightly higher levels of in vitro expression as compared with the cDNA construct. Similarly, one transgenic strain derived from the transgenic

construct with HSA intron 1 did express. The inclusion of introns 1 and 2 in vitro also resulted in higher expression than the cDNA. These two introns in transgenics resulted in a much better penetrance then either intron 1 or the cDNA. The inclusion of the first 6 introns (1-6) or the last 8 introns (7-14) or all of the introns (1-14) within the in vitro constructs resulted in a markedly higher level of expression. These in vitro results correlated with in vivo results where about 33% (HSA introns 1-6) , 43% (HSA introns 7-14) and 50% (HSA introns 1-14) of respective transgenic strains, generated from vectors with indicated HSA introns, express HSA in their milk and two or one strains, respectively, generated from vectors with introns 1-6 or 7-14 express at levels greater than 1 mg/ml. In addition, in vitro vectors possessing either HSA introns 2 + 7-14 or 1 + 2 + 12-14 support expression of even higher levels of HSA in vitro than the previous group of vectors. Similarly, 75% (3 of 4) of transgenic strains generated from a transgenic vector with HSA introns 2 + 7-14 and 100% (1 of 1 thus far tested) generated from a vector with introns 1 + 2 + 12-14 express HSA with one strain of each expressing high levels at about or above 1 mg/ml. While the number of transgenic strains thus far analyzed is limited, it appears that higher penetrance and/or levels of expression can be obtained in transgenics using vectors comprised of subsets of the introns of the HSA gene as opposed to the entire HSA gene with all of its introns. The production of transgenic goats (Example 24) with constructs demonstrated to express high levels of HSA in the milk of transgenic mice or with constructs which are the transgenic counterparts of the in vitro constructs which support very high levels of HSA, should result in very high levels of expression in the milk of these dairy animals.

In order to examine the tissue specificity of expression of HSA RNA total RNA was isolated from various tissues of transgenic female mice on day 10-12 of lactation. RNAs were fractionated by electrophoresis, transferred to nylon membrane and probed with a ^{32}P-labeled HSA antisense RNA as described in Example 16.

High levels of HSA mRNA were detected in the mammary gland of lactating females which secrete HSA into their milk (strain # 23) (Fig. 7). Transcripts are also found in skeletal muscle, kidney, liver and skin but not in other tissues (spleen, heart, salivary gland, lung and brain). The expression in liver appears to be endogenous mouse serum albumin . This ectopic

expression of transgene transcripts is not associated with any apparent physiological abnormality. Transgene transcripts from other genes fused to the 5'-flanking sequences of the genes of milk specific proteins have been shown to accumulate in non-mammary tissues such as salivary gland, kidney and brain.

The expression of HSA in RNA in the mammary tissue of transgenic strain #23 was also demonstrated by in situ hybridization as described in Example 16 and shown in Fig. 9.

The screening of transgenic mice for their expression of HSA in their milk was standardly performed on the milk of lactating females following parturition. The expression of HSA from explant cultures of mammary glands of both virgin and lactating transgenic strain #23 was also demonstrated (Example 17 and Fig. 9). In addition, we have shown a correlation between levels of expression of HSA in milk and levels of expression and secretion of HSA from mammary explants of virgin females among different strains of transgenic mice. Assay of explant cultures of mammary tissue of young transgenic goats or other form of dairy animals would greatly facilitate the identification of transgenic animals which will ultimately express HSA in their milk upon lactation.

Having now generally described the invention, a more complete understanding can be obtained by reference to the following specific examples. These examples are provided for the purpose of illustration only and are not intended to be limiting unless otherwise specified.

Example 1

A. CLONING OF THE SHEEP ß-LACTOGLOBULIN GENE WITH 5' AND 3'-FLANKING REGIONS (λ22-1, λ10-1)

A restriction map of the sheep ß-lactoglobulin (BLG) gene (Simons et al.., 1988, Bio/Technology 6; 179-183) indicated that the transcribed BLG sequences possessed only one EcoR I site and that EcoR I sites existed about 3 Kb upstream of the transcription region within 5'-flanking sequences and about 1 Kb downstream of the transcription unit within 3'-flanking sequences. Restriction digestion of the BLG gene and flanking regions would therefore

release 2 BLG gene fragments; (1) a fragment (approximately 4.1-4.3 Kb) made up of 5'-flanking sequences, including the BLG promoter, and the first part of the BLG transcription unit (designated 5'-region) and (2) a fragment (approximately 4.4 Kb) composed of the rest of the transcription unit including the polyadenylation site and 3'-flanking sequence (designated 3'-region). Therefore, the BLG gene and flanking regions were cloned as two gene halves made up of these two EcoR I subgenomic fragments.

High molecular weight sheep liver DNA was digested extensively with the restriction enzyme EcoR I. Restriction fragments were resolved on a 0.6% agarose gel along with size marker DNA standards. In order to identify the location of the BLG fragments on the gel, an analytical vertical strip of gel, made up of the size markers and about 5% of the restricted sheep DNA was cut from the rest of the gel, stained with Ethidium Bromide (EtBr) and photographed under UV light. The rest of the gel, the preparative portion, was wrapped in plastic wrap and put at 4°C until ready for use. The analytical portion of the gel was subjected to Southern analysis using a ^{32}P-labeled probe produced by the random primer method, with a bovine BLG cDNA as template. The bovine cDNA clone used as probe was kindly supplied by Dr. Carl A. Batt, Cornell University. Only a single band was detected of molecular weight of approximately 4.3 Kb. This single band represents both the 4.3 and 4.4 EcoR I subgenomic fragments which comigrated in this gel system. This comigration was verified in other Southern analyses using 4.3 and 4.4 Kb specific probes. The corresponding region of the preparative gel containing the BLG fragments was cut out of the agarose gel. DNA was electroeluted from this gel piece, purified by Eluptip-d (Schleicher and Schuell)(i.e. gel and elutip purified) and ethanol precipitated.

In order to isolate and clone the BLG fragments, the purified subgenomic DNA eluted from the gel was ligated into a Stratagene lambda-gt10 vector which had been previously digested with EcoR I and dephosphorylated. Ligation products were packaged with Gigapack plus extracts (Stratagene) and approximately 500,000 plaques of the resultant subgenomic library plated out on C600 cells (Stratagene) and incubated for plaque formation. Plates were lifted, in duplicate, onto nitrocellulose filters and treated by standard procedures. Filters were subsequently baked, prehybridized (5 x SSPE, 1/25 Blotto, 0.2% SDS) and hybridized with the same buffer containing ^{32}P-bovine

BLG cDNA probe at 62°C overnight. Filters were washed, the last most stringent wash was with 1 x SSC, 0.2% SDS at 62°C. Filters were subsequently subjected to autoradiography. Several duplicate positive plaques were identified and subplaqued to purity in Y1088 cells. Clones which contained either the 5'-region or the 3'-region of BLG were identified by differential hybridization with either BLG exon 2 and 5'-probes or exon 5 and 3'-probes, respectively. A clone possessing the 5'-region (5'-clone), designated λ22-1, as well as a clone possessing the 3'-region (3'-clone), designated λ10-1 were utilized for subsequent subclonings.

B. SUBCLONING BLG 5' AND 3' REGIONS FROM λ22-1 and λ10-1 PHAGE INTO pGEM-1 PLASMID (p570, p568)

Recombinant phage (λ22-1 and λ10-1) and DNA were purified using LambdaSorb® (Promega) using their protocols. Cloned BLG DNA were released from recombinant phage DNA by digestion with EcoR I. The released BLG 5'-region (approximately 4.3 Kb) from λ22-1 DNA and BLG 3'-region (approximately 4.1-4.4 Kb) from λ10-1 DNA were gel and elutip purified and ethanol precipitated.

Plasmid vector, pGEM-1, was prepared for the subcloning of the BLG 5'-region by digestion with Pvu II and EcoR I, and the large vector fragment was gel and elutip purified and ethanol precipitated. A cloning adapter was prepared by the annealing of two synthetic oligonucleotides as shown.

```
      SalI        (EcoR I)
     ------      ------
5'   GTCGACGCGGCCGC      3'
3'   CAGCTGCGCCGGCGTTAA  5'
```

(NOTE: Sites in parenthesis are not cleavable.)

The adapter was designed to allow the EcoR I compatible end to ligate to either of the EcoR I ends of the BLG 5'-region insert and by doing so to destroy the EcoR I site at that ligation junction, as the adapter sequence varied from an authentic EcoR I sequence by the incorporation of a G following the 5'-AATT instead of a C as would be found in the authentic sequence. Further, the adapter would link the adjoined BLG 5'-region to the Pvu II site of the prepared pGEM-1 vector by ligation of the blunt Pvu II site and blunt end of the adapter.

A BLG EcoR I end which did not ligate to an adapter was free to ligate to the EcoR I site of the prepared pGEM-1 vector. The prepared pGEM-1 vector, the BLG 5'-region insert fragment and the adapter were ligated in one step. The BLG DNA could ligate between the adapter and plasmid EcoR I sites in either of its two orientations. Ligation products were transformed into E. coli DH5 cells to ampicillin resistance. Resistant bacterial colonies were analyzed for the presence of plasmid containing BLG inserts by Whatman 541 filter lifts and hybridization with ^{32}P-labeled BLG exon 2 and 5' probes. Positive colonies were selected and grown in LB-amp medium. Plasmid DNAs were prepared from these isolates. Restriction analysis of clones with BamH I allowed for the selection of clones containing the desired orientation of BLG insert. BamH I digestion of plasmids with the desired BLG orientation resulted in DNA fragments of approximately 4200 and 2800 bp while DNA fragments from the non-desired orientation were approximately 5800 and 1380 bp. Restriction analysis confirmed that the desired clone contains the BLG 5'-region between the Pvu II and EcoR I sites of pGEM-1. The blunt end of the adapter is adjacent to the pGEM-1 Pvu II site. The Pvu II site is no longer cleavable at this location of the final clone. The 5'-end of the BLG 5'-region is linked to the EcoR I compatible region of the adapter. This EcoR I site is destroyed by the ligation. A Sal I site had been introduced with the adapter, just upstream of the 5'-end of the BLG region. This site is not found within either the BLG 5' or 3'-regions. The EcoR I site of the 3'-end of the BLG 5'-region linked to the pGEM-1 EcoR I site is regenerated and available for later digestion and ligation to the 5'-end of the BLG 3'-region. The desired correct BLG 5'-region construct (clone) selected for further work was designated p570. Clones with the BLG sequences in the undesired orientation were designated p571 and utilized in later constructions.

The EcoR I restricted, purified 4.4 Kb BLG 3'-region DNA was subcloned into plasmid pGEM-1 between the plasmid BamH I and EcoR I restriction sites. pGEM-1 digested with BamH I and EcoR I was gel and elutip purified, and ethanol precipitated. A cloning adapter made from two annealed synthetic oligonucleotides had the following sequence;

```
        (EcoRI)        BamHI
        ____                ____
5'   AATTAGCGGCCGCG          3'
3'       TCGCCGGCGCCTAG      5'
```

As with the previous adapter, it's EcoR I compatible end allowed for ligation with either of the EcoR I ends of the BLG 3'-region but resulted in loss of the EcoR I site. In this case the adapter EcoR I compatible site was followed by a T/A bp as opposed to a G/C bp as would be found in an authentic EcoR I sequence. A three part ligation with prepared pGEM-1 (BamH I and EcoR I ends), the adapter (BamH I and EcoR I compatible ends), and the BLG 3'-region insert (EcoR I ends) was performed. The BamH I site of the adapter should ligate to the BamH I site of pGEM-1. The BLG 3'-region would ligate between the adapter EcoR I compatible site and the EcoR I site of pGEM-1 in either orientation. Ligation products were introduced into E. coli DH5 cells by transformation to ampicillin resistance. Clones with the desired BLG 3'-region orientation were characterized by BamH I generated fragments of approximately 4500, 2000 and 800 bp. Restriction analysis of desired clones confirmed that the EcoR I site at the 5'-end of the BLG 3'-region was ligated to the EcoR I site of pGEM-1 and that this regenerated the EcoR I site; the EcoR I site at the 3'-end of the BLG 3'-region was ligated to the EcoR I compatible site of the adapter resulting in the destruction of this EcoR I site; the ligated BamH I sites of the adapter and pGEM-1 had regenerated a BamH I site; and that the Sal I site of the pGEM-1 polylinker was just downstream of the BLG 3'-region (and adapter). The EcoR I site at the 5'-end of the BLG 3'-region would be used later to join together the two BLG gene halves. A desired clone, designated p568, was selected for further work.

C. CONSTRUCTION OF A COMPLETE BLG VECTOR WITH A SnaB I SITE REPLACING THE Pvu II IN BLG EXON 1 (p585 vector)

In order to facilitate the cloning of foreign genes (such as HSA) into the correct Pvu II site of the BLG vector (within the untranslated portion of BLG exon 1), a SnaB1 site was introduced into this Pvu II site. No SnaB I site exists within natural BLG sequences or in the pGEM bacterial plasmid sequences in which the BLG sequences are cloned. Therefore, the introduced SnaB I site is unique, simplifying the introduction of foreign genes into the appropriate location of the BLG sequences.

The 5' portion of the BLG gene (EcoR I subgenomic) cloned into pGEM-1 (i.e. plasmid p570) possesses three Pvu II sites including the appropriate site within BLG exon 1. The other two Pvu II sites were mapped to locations

approximately 2100 bp and 2600 bp, respectively, upstream of the Pvu II site within exon 1. Plasmid p570 was partially digested with Pvu II and linearized plasmid (approximately 7200 bp) was gel and elutip purified. A synthetic SnaB I linker oligonucleotide of the sequence 5'-GTACGTAC-3' was self annealed. Annealed linker was ligated with the purified linearized plasmid p570. Ligation products were transformed into E. coli DH5 cells to ampicillin resistance. Desired recombinants were identified by the presence of a SnaB I site, the absence of the Pvu II site in exon 1 and the presence of the two upstream Pvu II sites. The correct plasmid was designated p583.

The 5'-half of the BLG gene with the SnaB I site replacing the Pvu II in exon 1 was recombined with the 3'-half of the BLG gene as follows. Construct p583 (containing the 5'-BLG region) was digested with Pvu I (within pGEM-1) and EcoR I (at the junction of the 3' end of the 5' BLG region and the adjacent pGEM-1) The DNA fragment (approximately 5800 bp) containing the 3'-portion of the plasmid Amp resistance gene, the plasmid ori, and the 5'-portion of the BLG gene was gel and elutip purified and ethanol precipitated.

The plasmid containing the 3'-half of the BLG gene, p568, was similarly digested with Pvu I and EcoR I. The DNA fragment of approximately 5800 bp containing pGEM-1 sequences including the 5'-portion of the Amp resistance gene up to its Pvu I site and complementary to those in the fragment described above, and the 3'-half of the BLG gene up to its 5' EcoR I junction with pGEM was gel and elutip purified and ethanol precipitated. The two purified fragments were ligated together and transformed into E. coli DH5 cells to ampicillin resistance. Only correctly recombined fragments would regenerate a complete ampicillin resistance gene. Correct recombination of a complete BLG gene was confirmed by restriction analysis by the release of the full length BLG region of approximately 8800 bp with restriction enzyme Sal I. This new plasmid, designated p585, is composed of a pGEM-1 bacterial plasmid and the complete BLG gene as described, with a SnaB I site substituting for the Pvu II site in exon 1 of BLG.

D. CLONING OF SHEEP GENOMIC SEQUENCES EXTENDING UPSTREAM OF THE 5'-BLG SEQUENCES IN PREVIOUS BLG VECTORS (p639, p642)

The 5'-sequences, upstream of the BLG coding sequence, within the previously discussed BLG vectors encompass a genomic DNA fragment of approximately 3 Kb. This 3 Kb region includes the BLG promoter sequences. In order to determine if sequences upstream of this 3 Kb fragment contain elements which would increase the consistency and/or level of expression from the BLG promoter, upstream genomic sequences were cloned.

High molecular weight sheep liver DNA was subjected to Southern analysis utilizing a variety of restriction enzymes. The very 5'-end of the BLG sequences already obtained, i.e. the Sal I to Pvu II fragment (approximately 450 bp) hybridized to repetitive sequences in genomic Southerns. Therefore, the Pvu II-Pvu II fragment (approximately 500 bp) downstream of the Sal I-Pvu II fragment and the Pvu II-BamH I fragment (approximately 600 bp) downstream of the Pvu II-Pvu II fragment were used as a probe. These probe fragments were generated by digestion of p570 with BamH I and Pvu II, gel and elutip purification of the appropriate 500 and 600 bp fragments, and ethanol precipitation. Probe DNA was ^{32}P-labeled by the random primer technique.

Southern analysis revealed a probe positive Hind III fragment (approximately 8 Kb) and a probe positive Sac I fragment (approximately 8.6 Kb). This allowed us to map Hind III and Sac I sites to approximately 2.5 Kb and 7.8 Kb, respectively, upstream of the 5'-end (EcoR I site) of the previously obtained BLG sequences.

High molecular weight sheep DNA was digested extensively with either Sac I or Hind III. Restriction fragments were resolved on 0.6% agarose gels. An analytical strip of each gel, made up of size markers and about 5% of the restricted sheep DNA was cut from the rest of the gel, stained with ethidium bromide and photographed under UV light. The rest of the gels, the preparative portions, were wrapped in plastic wrap and put at 4°C until ready for use. The analytical portions were subjected to Southern analysis using the ^{32}P-labeled probe discussed above. The probe positive Hind III (8 Kb) and Sac I (8.6 Kb) fragments were identified. Corresponding regions were cut out of the preparative portions of the gels. DNAs were electroeluted, elutip isolated and ethanol precipitated.

Vector Lambda Zap II (Stratagene) was used for construction of subgenomic libraries for both the Hind III and Sac I generated fragments. For the Sac I subgenomic library, Lambda Zap II was digested with Sac I and dephosphorylated with CIP. For the Hind III subgenomic library, Lambda Zap II was first self ligated in order to ligate cos ends together. It was subsequently digested with Spe I and partially filled in with Klenow polymerase and dCTP and dTTP.

The purified Hind III subgenomic DNA was partially filled with Klenow polymerase and dGTP and dATP. These partially filled Hind III ends were compatible with the partially filled Lambda Zap II Spe I ends. The Hind III subgenomic DNA was ligated into the Spe I digested λ vector and the Sac I subgenomic DNA was ligated into the Sac I digested λ vector. Each was packaged with Gigapack Plus II extracts (Stratagene) and plated on PLK-A cells (Stratagene). Plates were lifted, in duplicate, onto nitrocellulose filters. Filters were treated as discussed in the library screen for λ22-1 and λ10-1. Filters were hybridized with the ^{32}P-labeled probe discussed earlier in this section. Filters were washed. Duplicate positive plaques were subplaqued to purity with the final, most stringent wash being with 0.5xSSC, 0.2%SDS, 62°C. The pBlueScript SK phagemid containing BLG sequences were in vivo excised from the Lambda Zap II positives clones using R408 helper phage (Stratagene) by the method recommended by the supplier. The phagemids were rescued on XL1-Blue cells (Stratagene) and selected on LB-ampicillin plates. Selected colonies were cultured in LB-ampicillin medium and plasmids from these cultures were subjected to restriction analysis.

Correct clones of the Hind III subgenomic cloning were initially identified by the presence of an approximate 4.4 Kb EcoR I fragment and approximately 1900 and 1075 bp Asp 718 (isoschizomer of KpnI) fragments all of which had been previously delineated by mapping of the BLG region already cloned. Both orientations of the BLG Hind III subgenomic fragment cloned into the Spe I site of the vector were found. The desired orientation with the 5'-end of BLG Hind III fragment cloned into the Spe I site towards the plasmid multiple cloning Sal I site was characterized by the release of a fragment of approximately 2500 bp upon digestion with EcoR I and Hind III. This desired clone of the Hind III subgenomic DNA was designated p639. The 5'-end of the BLG region was

extended approximately 2.5 Kb further upstream from the 5'-end of the original BLG clone (e.g. p585).

Correct clones of the Sac I subgenomic cloning were initially identified by the release of a fragment of approximately 2500 bp upon digestion with EcoR I and Hind III and a 3800 bp fragment upon digestion with Sac I and Hind III. The clones with the desired orientation of BLG sequences within the pBlueScript SK plasmid were identified as follows. Sac I subgenomic clones were digested with EcoR I and subjected to Southern analysis using the ^{32}P-labeled p570 BamH I/Pvu II probe homologous to the 5'-end of the p570 BLG sequences and therefore to the 3'-end of the BLG sequences within the Sac I subgenomic fragment described above. A probe positive EcoR I fragment of approximately 4.2 Kb identified the plasmid as being the desired orientation with the upstream most end of the Sac I fragment being next to the plasmid multiple cloning site including the Sal I site. This correct, desired clone was designated p642.

Sequencing confirmed that p639 (Hind III subgenomic) and p642 (Sac I subgenomic) contained BLG upstream sequences which overlapped the original BLG clones (e.g., p571). The 5'-sequence of the original BLG 5'-portion cloned in p571 was determined using an Sp6 promoter primer of the sequence, 5' ATTTAGGTGACACTATA 3'. The sequence was further extended into the BLG sequences of p571 by using a primer, 5' TGTTTGGGGACTTCCCTGGTGA 3', derived from sequence obtained using the Sp6 promoter primer.

The sequences obtained using the second primer were identical in the p571, p639 and p642 construction confirming that the latter two clones contained BLG upstream sequences. In addition a third primer, 3' AGTCCCACTACGACCGGAG 5', derived from sequence obtained from the Sp6 promoter primer was used to obtain sequence upstream of the 5'- EcoR I site in the original BLG clone. As expected sequences obtained from both p639 and p642 were identical and the proximal most 25 bases were identical with the 5'-most bases of p571 and contained the natural EcoR I site.

E. CONSTRUCTION OF COMPLETE BLG VECTORS WITH BLG CODING REGIONS WITH EXTENDED 5'-SEQUENCES (p644, p646)

The 5'-extended BLG sequences cloned as Hind III and Sac I sheep DNA subgenomics in plasmids p639 and p642, respectively, were incorporated into full length vectors capable of expressing BLG as follows.

For the incorporation of sequences contained within the Hind III fragment, the 5'-extension was first recombined with the 5'-BLG region in plasmid p590. p590 was digested to completion with Asp 718 and partially with Sal I. The DNA fragment of approximately 4940 bp which included BLG sequences downstream of the Asp 718 site through the Sal I site and adjacent pGEM sequences was gel and elutip purified and ethanol precipitated. The 5'-end of the BLG Hind III fragment (approximately 4600 bp) was released from p639 by digestion with Sal I (in the polylinker, just upstream of the 5'-end of the Hind III fragment), and Asp 718 (downstream of the original BLG 5'-EcoR I site), gel and elutip purified, and ethanol precipitated. The prepared p590 and p639 fragments were ligated together. Ligation products were transformed into E. coli DH5 cells to ampicillin resistance. Correct recombinants were diagnosed by the fact that restriction by EcoR I produced two fragments (approximately 7090 and 2500 bp), Sal I produced two fragments (approximately 6758 and 2835 bp) and Bgl II linearized plasmid to the size of approximately 9590 bp. Correct clones were designated p640. Plasmid p640 contains a BLG 5'-region of approximately 5.5 Kb from the upstream Hind III site to the transcriptional initiation site just upstream of the SnaB I cloning site. This 5.5 Kb 5'-region was recombined with complete BLG coding and 3'-regions. Plasmid p640 was digested with Pvu I (within the pGEM) and SnaB I releasing a DNA fragment of approximately 7043 bp made up of part of the pGEM vector and the 5.5 Kb BLG 5-region. Plasmid p585 was also digested with Pvu I and SnaB I releasing a DNA fragment (approximately 7041 bp) comprised of the rest of pGEM and the BLG coding and 3'-regions. These fragments were gel and elutip purified and ethanol precipitated and subsequently ligated together. Ligation products were used to transform DH5 cells and positive transformants selected on LB-ampicillin plates. Hind III digestion resulting in 3 DNA fragments (approximately 7840, 3410 and 2830 bp) identified correct recombinant clones consisting of 5'-BLG sequences (approximately 5.5 Kb), BLG coding sequences and 3'-BLG sequences. Correct clones were designated p644.

The 5'-BLG sequences contained within the sheep DNA Sac I clone, p642, were incorporated into a full length BLG vector by first joining these sequences to the 5'-BLG sequences contained within plasmid p640 which possesses 5'-BLG sequences derived from the Hind III clone p642. Plasmid p640 was digested with EcoRV which restricted it at 2 sites. One EcoRV site was contained within the pGEM polylinker just upstream of the 5'-end of the Hind III BLG sequences. The second EcoRV site was just upstream of the original 5'-BLG EcoR I site. The resultant DNA fragment of approximately 7150 bp was gel and elutip purified and ethanol precipitated. The ends of this fragment were then dephosphorylated with calf intestinal alkaline phosphatase (CIP) (Promega). Into this dephosphorylated EcoRV site was ligated BLG sequences which extended from the common EcoRV site just upstream of the original 5'-EcoR I site up to the EcoRV site. This latter DNA fragment (approximately 7700 bp) was obtained by digestion of p642 with EcoRV and subsequent gel and elutip purification and ethanol precipitation. Ligation products were transformed into DH5 cells and positive transformants selected on LB-ampicillin plates. Clones with the correct, desired orientation of the p642 insert into p640 were characterized by the presence of two Bgl II DNA fragments (approximately 10,140 and 4710 bp). A Bgl II site had been mapped to about 600 bp downstream of the 5'-Sac I site by Southern analysis of sheep DNA. Correct clones were designated p645. p645 clones contain 5'-BLG sequences of approximately 10.8 Kb upstream of the BLG transcriptional initiation site, followed by the SnaB I site, the 3'-BLG region containing the native BLG polyadenylation signal and site and the pGEM bacterial plasmid. p645 does not contain BLG coding sequence. p645 was used to produce a full length BLG vector with 10.8 Kb 5'-BLG sequences. p645 was digested with SnaB I and Pvu I (within pGEM). The DNA fragment of approximately 12,290 bp, made up of bacterial plasmid sequences as well as all 10.8 Kb 5'-sequences upstream of the SnaB I site, was gel and elutip purified and ethanol precipitated. The BLG coding sequence and BLG 3'-sequences as well as bacterial plasmid were obtained as a SnaB I, Pvu I DNA fragment (approximately 7040 bp) from p585 similarly prepared by gel purification. These two DNAs were ligated together and transformed into E. coli DH5 cells to ampicillin resistance. Correct recombinants produced DNA fragments of approximately 14,040, 2860 and 2430 bp upon Xba I digestion and were designated p646.

Example 2

CLONING THE HUMAN SERUM ALBUMIN (HSA) GENE (p650, p651)

The DNA sequence of the HSA gene was determined by Minghetti et al.. (J. Biol. Chemistry 261; 6747-6757, 1986) and entered into the NIH nucleic acid sequence data bank GenBank 67. Three Nco I sites within this sequence were identified using the SEQ-Sequence Analysis Systems program of IntelliGenetics, Inc. The first Nco I site lies about 275 bp upstream of HSA exon 1. The second site lies within exon 7 and the third site lies about 227 bp downstream of exon 15. Therefore, digestion of human high molecular DNA with Nco I should release two fragments of 8079 and 9374 bp which together encompass the entire HSA gene. The 8079 bp fragment represents the 5'-half of the HSA gene while the 9374 bp fragment represents the 3'-half of the gene. The strategy to clone out the gene was to digest human DNA with Nco I, to make 2 separate subgenomic libraries from DNAs of the approximate sizes of the 2 expected HSA fragments and to identify HSA clones from these libraries.

High molecular weight human placental DNA was digested extensively with Nco I. Restriction fragments were resolved on a 0.6% agarose gel along with size marker DNA standards. An analytical vertical strip of the gel, made up of the size markers and about 5% of the restricted human DNA was cut from the rest of the gel, stained with ethidium bromide and photographed under UV light. The rest of the gel, the preparative portion, was wrapped in plastic wrap and put at 4°C until ready for use. The analytical portion of the gel was subjected to Southern analysis using digoxigenin-dUTP labeled HSA cDNA probe produced by the random primer method according to protocols supplied with the Genius System DNA Labeling Kit (Boehringer Mannheim). The substrate DNA for the production of the probe was the HSA cDNA sequence released from plasmid pHSA-F1$^-$ (see below) by digestion with Sal I and EcoR I. Detection of probe positive bands of correct size (approximately 8079 and 9374 bp) in the Southern analysis were identified using the Genius Nucleic Acid Detection Kit (Boehringer Mannheim), Lumi-Phos 530 (Boehringer Mannheim) and autoradiography according to manufacturers instructions. The individual corresponding regions of these HSA fragments were cut out of the preparative part of the agarose gel. DNA was electroeluted, elutip purified, and ethanol precipitated.

The 2 purified Nco I subgenomic fractions were individually ligated into Lambda ZapII vector, digested with EcoR I and dephosphorylated with alkaline phosphatase (Stratagene, Inc.) using 2 synthetic oligonucleotides which when annealed form an adaptor of the structure:

```
                          Nco I       NruI        EcoR I
Oligonucleotide A:  5'  CATGGCAACGATCGCGAGTCGACG      3'

Oligonucleotide B:  3'      CGTTGCTAGCGCTCAGCTGCTTAA  5'
                                        Sal I
```

Prior to annealing the 5'-end of oligonucleotide B was phosphorylated using T4 polynucleotide kinase by standard procedures, so as to supply a 5'-phosphate group required for the ligation of the EcoR I site of the adaptor to the dephosphorylated EcoR I site of the lambda vector. The 5'-phosphate group of the Nco I site of the subgenomic human DNA fractions allows ligation of this site to the nonphosphorylated Nco I site of the adaptor. Ligation products were packaged into phage using Gigapack II Plus packaging extract (Stratagene, Inc.) according to the suppliers protocols.

Libraries were produced by the adsorption of packaged phage into PLK-A cells (Stratagene, Inc.) and plated. Approximately 100,000 plaques of the library produced from the Nco I subgenomic fraction containing the HSA 8079 bp fragment and 50,000 plaques of the library produced from the Nco I subgenomic fraction containing the HSA 9374 bp fragment. Plates were lifted in duplicate onto nitrocellulose filters. Filters were treated by standard procedures, prehybridized (5 x SSPE, 1/25 Blotto, 0.2% SDS) and hybridized overnight at 62°C with the same buffer containing the ^{32}P-labeled HSA cDNA probe discussed above. Filters were washed and subjected to autoradiography. Duplicate positive plaques were identified and subplaqued to purity. The pBluescript SK plasmid component of the Lambda ZapII vector, containing HSA gene inserts, were in vivo excised from the vector using the R408 helper phase supplied by Stratagene, Inc. according to their protocols. Plasmids containing the 5'-half of the HSA gene were designated p651. Plasmids containing the 3'-half of the HSA gene were designated p650.

Restriction analysis of p650 confirmed that this clone in fact represented the insertion of the Nco I 5'-half of the HSA gene into the EcoR I site of

pBluescript SK. Digestion of p650 with the following restriction enzymes yielded the expected DNA fragments.

Restriction Enzyme	Expected & Observed DNA Fragments (bp)
Bgl II	5363; 4921; 754; 47
BstEII	11085
EcoR I	3541; 2958; 2008; 1603; 709; 266
Hind III	6564; 4281; 240
Nco I	8079; 3006
Nco I + BstEII	7756; 3006; 323
ScaI	2511; 2449; 2405; 2368; 1352
Xba I	6926; 1882; 1296; 981

The expected locations of the restriction sites were derived from a restriction map of pBluescript SK (Stratagene, Inc.) and the SEQ restriction analysis program of Intelligenetics, Inc. of the sequence of the appropriate region of the HSA gene as published by Minghetti et al.. (1986, J. Biol. Chem. 216:6747-6757). The restriction analysis of p650 also determined that the HSA gene region was cloned into the pBluescript vector with its 5'-end toward KpnI site of the vector multiple cloning site.

It had been expected that the restriction of p651 with Nco I would result in 2 fragments of 9374 and 3006 bp; the former representing the Nco I 3'-half of the HSA gene and the latter representing the pBluescript sequences into which the HSA sequences were cloned. However, while the 3006 bp (pBluescript) band was visible, the 9374 bp band was not found. Instead a band of approximately 4000 bp was present. In order to determine whether this band represented HSA sequences, p651 was subjected to sequencing using several sequencing primers (synthesized in house) using either the Sequenase Sequencing kit (United States Biochemical Corp.) or the T7Sequencing kit (Pharmacia) according to manufacturer's protocols. The EcoR I site within pBluescript into which the cloned sequences were introduced is flanked by a T7 promoter on one side and a T3 promoter on the other side. We therefore used T7 (sense) and T3 (antisense) sequencing primers in order to sequence the termini of the cloned sequences. HSA exon 7, 8, 9, and 11 specific primers (sense) as well as two exon 12 specific primers (sense) and an

exon 15 specific primer (antisense) were also used. Primer sequences were as follows:

Primer	Sequence	Corresponding HSA gene bp position#
T7	5' AATACGACTCACTATAG 3'	
T3	3' GAAATCACTCCCAATTA 5'	
HSA Exon 7	5' CATGGAGATCTGCTTGAA 3'	9541 - 9558
HSA Exon 8	5' GACTTGCCTTCATTAGCT 3'	10996 - 11013
HSA Exon 9	5' GAGAAGTGCTGTGCCGCT 3'	12566 - 12583
HSA Exon 11	5' GTACCCCAAGTGTCAACT 3'	15002 - 15019
HSA Exon 12(A)	5' GACAGAGTCACCAAATGC 3'	15588 - 15605
HSA Exon 12 (B)	5' GAGAGACAAATCAAGAAAC 3'	15735 - 15753
HSA Exon 15	3' AGTCGGATGGTACTCTTATTCTC 5'	18522 - 18544

Specific sequence information from all primers except the HSA exon 8,9 and 11 specific primers was obtained suggesting that these latter regions were lacking in p651. Sequences obtained from sequencing reactions using the primers corresponded to HSA gene sequences as indicated below.

Primer	Sequence obtained from primers corresponding to HSA gene bp position and region	
T7	9540 - 9737	(Part of exon 7 and into intron 7)
T3	18919 - 18669	(3'-flanking region of exon 15 and into exon 15)
HSA exon 7	9605 - 9616	(Intron 7)
HSA exon 12(A)	15681 - 15690	(Exon 12)
HSA exon 12(B)	15796 - 15807	(Intron 12)
HSA exon 15	18497 - 18156	(Intron 14)

Clearly, the 5'- and 3'-ends of the 3'-half of the HSA gene, including Nco I sites at each end, as well as exon 7 and at least part of intron 7 from the 5'-end and the 3'-sequences flanking exon 15 and exon 15 through exon 12 from the 3'-end. These results demonstrate that the 3'-half of the HSA gene was present in p651, but that an internal deletion of HSA sequences had occurred. The deletion was also found in the parent Lambda phage. Therefore, construct p651 contains the HSA gene from exon 12 through exon 15 and beyond into 3'-flanking sequences to the Nco I site, including introns 12, 13 and 14.

Restriction analysis of p651 demonstrated that the Asp I site within exon 12 was present.

In order to clone the HSA gene sequences between exon 7 and exon 12 so as to obtain that region which includes introns 7-11, we utilized PCR technology. Four PCR reactions were set up using synthetic priming oligonucleotides homologous to desired regions of the HSA gene containing useful restriction sites. The PCR reactions were designed so that the upstream end of reaction #1 overlapped the Nco I site within exon 7. The downstream end of reaction #1 overlapped the Avr II site within intron 8. This same Avr II site was overlapped by the upstream end of PCR reaction #2. The downstream end of PCR reaction #2 overlapped the Hind III site within intron 8. This Hind III site was also overlapped by the upstream end of PCR reaction #3. The downstream end of PCR reaction #3 overlapped the XhoI site in intron #10 which was also overlapped by the upstream end of PCR reaction #4. The downstream end of PCR reaction #4 overlapped the Asp I site in exon 12. By this PCR strategy the entire region desired would be obtained and adjacent PCR products would be joined together using overlapped restriction sites. PCR primers and reactions were as follows:

PCR #1

BamHI Nco I

Sense primer 5' GAATTCGGATCCCCATGGAGATCTGCTTGAATGTGCT 3'

* *

9540 9564

AvrII

Antisense primer 3' TGTTGAATCCTCCTATCGGATCCcagctg 5'

* *

11127 11149

PCR #2

AvrII

Sense primer 5' gtcgacCCTAGGCTTTTCTGTGGAGTTGCT 3'

* *

11144 11167

Hind III

Antisense primer 3' TTCAGGAATCGATGATTCGAActtaag 5'

* *

12339 12359

PCR #3

Hind III

Sense primer 5' gaattcAAGCTTTACTGCATGGGGTTTAGT 3'

* *

12354 12377

XhoI

Antisense primer 3' TGTTCTGTATCAAAGAAAGGAGCTCcagctg 5'

* *

14454 14478

PCR #4

XhoI

Sense primer 5' gtcgacCTCGAGTAGATTAAAGTCATACA 3'

* *

14473 14495

AspI BamHI

Antisense primer 3' TTGCGGTCATTCACTGTCTCAGcctaggcttaag 5'

* *

15575 15596

Note

Restriction sites in bold are found within HSA sequences. Non-HSA sequences are shown as small letters. Asterisks and associated numbers mark the corresponding HSA gene bp position.

PCR reactions were performed with high molecular weight human placental DNA as substrate [40 cycles of 94° C (1', 30"), 60° C (2'), 72° C (4')]. A sample of each reaction was analyzed on a 1% agarose gel. DNA bands of expected size (PCR #1, 1628 bp; PCR #2, 1228 bp; PCR #3, 2136 bp; PCR #4, 1142 bp) were seen. The remainder of the reactions were extracted two times with chloroform, 2 times with phenol/chloroform , 2 times with chloroform and subsequently ethanol precipitated. Products of reactions #1 and #2 were digested with Avr II. Products of reactions #3 and #4 were digested with XhoI. DNAs were subjected to agarose gel electrophoresis and DNA bands cut from the gel, electroeluted and elutip purified. The purified products of reactions #1 and #2 were ligated together and products of reactions #3 and #4 were ligated together. Since each PCR product had available 5'-phosphates only at their digested ends (Avr II or XhoI) only these ends were available for ligation and the other end of each DNA was not available for ligation. Ligation products of reactions #1 and #2 as well as ligation products of reactions #3 and #4 were subsequently each digested with BamH I and Hind III. The resultant 2814 bp DNA representing the ligation of reactions #1 and #2 at their common Avr II site with a digested BamH I site at its upstream terminus and a digested Hind III site at its downstream terminus was gel and elutip purified. This DNA was ligated into the BamH I and Hind III sites of plasmid vector pGEM-1. The 3243 bp DNA representing the ligation of reactions #3 and #4 at their common XhoI sites, with its upstream terminus digested at its Hind III site and downstream terminus digested at its BamH I site was similarly purified and ligated into the Hind III and BamH I sites of plasmid vector pGEM-2. Ligation products were introduced into MC1061 bacterial host cells by electroporation (BTX Electroporation System 600) according to manufacturer's protocols. Positive transformants were selected on ampicillin plates. Restriction analysis of DNAs from selected transformants identified desired clones. Construct p679 was the designation of the HSA PCR #1 and #2 products cloned into pGEM-1. It contains HSA sequences extending from the Nco I site in exon 7 (HSA gene bp position 9541) to the Hind III site in the downstream end of intron 8 (HSA gene bp position 12355). The pGEM-2 construct containing HSA PCR #3 and #4 products was designated p676. It contains HSA sequences extending from the same Hind III site in the downstream end of intron 8 (HSA gene bp position 12355) to the Asp I site in exon 12 (HSA gene bp position 15592).

When taken together with construct p650, containing the HSA gene sequences from the Nco I site upstream of HSA exon 1 (HSA gene bp position 1462) to the Nco I site in exon 7 (HSA gene bp position 9541) and construct p651, containing HSA gene sequences extending from upstream of the Asp I site in exon 12 (HSA gene bp position 15592) to the Nco I site downstream of exon 15 (HSA gene bp position 18915) these constructs, p679 and p676, complete the cloning of the entire HSA gene.

Example 3

COMPLETE BLG VECTOR WITH THE HSA cDNA UNDER THE CONTROL OF THE BLG PROMOTER (p575)

Vectors based upon use of the BLG promoter and gene system incorporate the foreign gene to be expressed (HSA) into the Pvu II site of the BLG gene within exon 1 just upstream of the BLG translational initiation ATG codon. There are a number of Pvu II sites within the BLG sequences. The BLG 5'-region cloned into pGEM-1 (i.e. plasmid p570) possesses three Pvu II sites including the appropriate site within BLG exon 1. The other two Pvu II sites were located approximately 2100 and 2600 bp, respectively, upstream of the Pvu II site within exon 1. In order to introduce the HSA cDNA into the appropriate Pvu II site of a complete BLG vector, the cDNA was first cloned into the correct site within the BLG 5'-clone (p570) and subsequently the BLG 5'-clone with HSA cDNA was joined to the BLG 3'-clone. Plasmid, p570, was partially digested with Pvu II. Linearized plasmid (approximately 7200 bp) was recovered by gel and elutip purification and ethanol precipitation.

The HSA cDNA was isolated from a lambda gt11-human liver cDNA library. The complete cDNA clone is 1,983 bp in length and contains the complete HSA coding sequences, including the 18 amino acid prepeptide, the 6 amino acid propeptide, and the 585 amino acids of the mature protein. The cDNA clone also contains 20 bp of 5'-untranslated and 141 bp of 3'-untranslated sequence. The cDNA clone was subcloned into the plasmid vector pBS(-) (Stratagene) between the vector BamH I and EcoR I polylinker restriction sites with the BamH I site at its 5'-end and EcoR I site at its 3'-end. This plasmid was referred to as pHSA-F1$^-$. The HSA cDNA sequence was released from the bacterial vector by restriction with BamH I and EcoR I. These

staggered ends were blunted with Klenow polymerase in the presence of excess dNTPs. The blunt ended HSA cDNA was ligated into the purified Pvu II linearized plasmid p570 discussed earlier. Ligation products were introduced into DH5 cells by transformation to ampicillin resistance. Desired recombinants were diagnosed by the presence of two Hind III restriction fragments (approximately 7807 and 1293 bp) and three BamH I restriction fragments (approximately 4280, 3170, and 1700 bp). This confirmed that the HSA cDNA was introduced into desired Pvu II site of BLG, in exon 1, in the same orientation as the BLG coding sequence and that the junction between the BLG Pvu II site and the Klenow blunted 5'-HSA BamH I site regenerated a functional BamH I site. Additional analyses verified that the junction between the 3'-HSA EcoR I site blunted with Klenow polymerase and the BLG Pvu II had regenerated a function EcoR I site. This correct plasmid clone was designated p572.

The 5'-region of the BLG gene with the HSA cDNA cloned into its Pvu II site in exon 1 was recombined with the 3'-region of the BLG gene as follows. Construct p572 was digested to completion with Pvu I (within pGEM) and partially with EcoR I. The desired fragment of approximately 7800 bp containing pGEM sequences and the 5'-region of the BLG gene to the junction of its 3'-end (EcoR I) with the pGEM-1 was gel and elutip purified and ethanol precipitated. This was ligated to the 3'-region of the BLG gene, released from plasmid p568, as a Pvu I/EcoR I fragment of approximately 5800 bp. This latter fragment contains pGEM-1 sequences up to its Pvu I site, and the 3'-region of the BLG gene up to its 5'-junction (EcoR I) with pGEM. Ligation products were used to transform DH5 cells to ampicillin resistance.

Correct, desired recombinants were characterized by Hind III fragments of approximately 7840, 3500 and 2200 bp and BamH I fragments of approximately 4760, 4244, 2000, 1700 and 870 bp. Correct recombinants were designated p575. These represent the complete BLG sequences utilized in the vectors including 5' and 3' regions, with the HSA cDNA introduced into the BLG Pvu II site just upstream of the BLG ATG translational initiation codon, in the same orientation as the BLG coding sequences, within a pGEM plasmid. BLG/HSA and HSA/BLG junctions were confirmed by sequence analysis. The BLG/HSA sequences are flanked by Sal I sites.

As Sal I sites are not found anywhere else in the BLG/HSA sequences, digestion of p575 with Sal I releases a BLG/HSA fragment, from the pGEM, suitable for injection into mammalian oocytes for the production of BLG/HSA transgenics.

Example 4

INTRODUCTION OF AN HSA MINIGENE CONTAINING THE FIRST HSA INTRON INTO A COMPLETE BLG VECTOR (p599)

In order to elucidate the HSA intron pattern which would result in high level expression of HSA in the milk of transgenics, HSA minigenes were constructed composed of the HSA cDNA with different combinations of its introns. One such minigene involves the incorporation of the first HSA intron into an HSA cDNA. In order to make such a construct, a Cla I restriction site was introduced into the region of the HSA cDNA which is derived from HSA exon 2, without changing its coding sequence. This was accomplished by in vitro mutagenesis (Amersham kit) using single stranded DNA template derived from pHSA-F1⁻ and a synthetic oligonucleotide of the sequence,

```
                    Arg
5' - GGTTGCTCATCGATTTAAAGATTTGGG-3'
                  Cla I
```

This mutagenesis introduced the Cla I site by replacing the HSA cDNA G with an A in the third base position of the codon for arginine, the 34th amino acid of the HSA protein (including the prepro sequence). Both CGA and CGG codons encode arginine. The resultant clone, made up of an HSA cDNA containing an introduced Cla I site within its exon 2 derived region in the pBS vector, was designated p595.

A DNA fragment composed of intron 1 of the HSA gene, along with parts of flanking exon 1 and exon 2, was produced by PCR technology using synthetic oligonucleotide primers which included sequences complementing to exon 1 and exon 2 as seen below.

Exon 1 Sense Primer

Hind III Met BstEII

5'-GTACATAAGCTTTGGCACAATGAAGTGGGTAACCTT-3'

Exon 1 sequence

Exon 2 Antisense Primer

Cla I

3'-CCAACGAGTAGCTAAATTTCTAAACCC-5'

*

Exon 2 sequence

*Introduced Cla I site by incorporating a T in this position rather than a C. No change in coding as discussed above.

The template for this PCR reaction was a clone of the HSA gene extending from the gene exon 1 to exon 3, including introns 1 and 2. This clone designated p594 will be discussed later. Following ethanol precipitation, the PCR product of 844 bp was digested with Bst EII and Cla I. The subsequent DNA fragment of 799 bp was gel and elutip purified and ethanol precipitated.

Plasmid p595 was digested with BstEII and Cla I. The large fragment lacking the BLG cDNA region between the BstEII and Cla I sites was gel purified, electroeluted, elutip isolated and ethanol precipitated.

The purified PCR product with BstEII and Cla I ends and the purified p595 fragment with BstEII and Cla I ends were ligated together. Ligation products were transformed into DH5 cells and positive transformants selected on LB-ampicillin plates.

Transformants containing plasmid containing the PCR insert were identified by colony lifts of plates using Whatman 541 filters as described earlier. The probe was ^{32}P-labeled random primer product of the PCR product used in the ligation. Following autoradiography of filters, probe positive colonies were picked, grown in LB-ampicillin medium. Plasmid preparations from these were analyzed separately with Cla I and Xba I. Correct plasmids which possessed a Cla I site and produced Xba I fragments of 4010 and 1874

bp were identified. A correct recombinant made up of an HSA minigene comprised of its cDNA and HSA intron 1 within a pBS vector was designated p596.

The HSA (intron 1) minigene was introduced into BLG vector p585 as follows: The HSA (intron 1) minigene was released from the pBS vector by complete digestion with BamH I and partial digestion with EcoR I. The 2701 bp minigene was gel and elutip purified and ethanol precipitated. The BamH I and EcoR I ends of the minigene were blunted using Klenow polymerase and excess dNTPs. This blunted fragment was ligated into BLG vector p585 which had been digested with SnaB I and dephosphorylated with CIP. Ligation products were transformed into DH5 cells to ampicillin resistance. Positive colonies were identified by colony lifts with Whatman 541 filters and hybridization to the HSA intron 1 PCR probe describe above. Probe positive colonies identified by autoradiography were grown in LB-ampicillin medium from which plasmid preparations were made. Clones which possessed the HSA (intron 1) minigene in the desired orientation, that is with the HSA minigene in the same orientation as the BLG gene, were identified by their release of restriction fragments of approximately 10676 and 3600 bp upon EcoR I digestion and approximately 4490, 3600, 3400 and 2860 bp upon EcoR I/Sal I double digestion. This desired clone, the HSA (intron 1) minigene in the correct orientation within the SnaB I site of BLG vector p585 was designated p599.

Example 5

A. CONSTRUCTION OF A BLG VECTOR LACKING CODING SEQUENCE (p590)

The full length BLG vector was altered so that while it still contains the 5'-BLG sequences, including promoter, upstream of the BLG coding sequence and 3'-BLG sequences, including the BLG polyadenylation signal and site, downstream of the coding sequence, all BLG coding sequence was deleted. Plasmid p583, containing the 5'-region of BLG with a SnaB I site replacing the Pvu II site in BLG exon 1, was digested with SnaB I and Pvu I (within pGEM). The DNA fragment (approximately 4490 bp) containing pGEM sequences and

the 5'-portion of the BLG gene up to the SnaB I site, was gel and elutip purified and ethanol precipitated.

Restriction mapping of plasmid p568, BLG 3'-portion in pGEM-1, demonstrated that the Xma I (isoschizomer of SmaI) site within exon 7 of the BLG gene was the 3'-most Xma I site within the 3'-portion of the gene. Plasmid p568 was digested with Xma I and Pvu I (within pGEM). The DNA fragment of approximately 2660 bp containing pGEM sequences and the 3'-end of the 3'-portion of the BLG gene (up to the Xma I site within exon 7) was gel and elutip purified and ethanol precipitated.

Two synthetic oligonucleotides were produced that when annealed formed a SnaB I/Xma I adaptor of the sequence.

```
  1
 2SnaB I      XmaI
5' - GTAGATCTC     3'
3' - CATCTAGAGGGCC 5'
       Bgl II
```

A ligation between the purified fragments from p583, p568 and the annealed oligonucleotide adaptor was performed. Ligation products were transformed into DH5 cells to ampicillin resistance. Correct recombinants were identified by the fact that Bgl II digestion linearized the plasmid, that BamH I digestion resulted in 3 fragments (approximately 4200, 2050 and 900 bp) and that SnaB I and Hind III digestion produced fragments of approximately 5950 and 1200 bp. These correct recombinants containing the 5'- and 3'-ends of the BLG gene connected by a SnaB I site but without BLG coding sequence were designated p590.

B. CONSTRUCTION OF BLG VECTOR (BLG 5-AND 3'-SEQUENCE, NO BLG CODING SEQUENCE) WITH AN HSA MINIGENE CONTAINING HSA INTRON 1 (p600)

Construct p590 was digested with SnaB I and resultant restricted ends dephosphorylated with CIP. The HSA minigene with intron 1 was released from p596 by complete digestion with BamH I and partial digestion with EcoR I. The 2701 bp fragment composed of the entire minigene was gel and elutip

purified and ethanol precipitated. The BamH I and EcoR I ends of this fragment were made blunt using Klenow polymerase and excess dNTPs.

The prepared minigene was ligated into the prepared p590 vector. Ligation products were introduced into E. coli DH5 cells by transformation to ampicillin resistance. Plates containing selected colonies were lifted onto Whatman 541 filters and processed as before. A ^{32}P-labeled probe, the PCR produced HSA intron 1 discussed in the section describing the construction of plasmid 599, was made by the random primer technique. Hybridization and subsequent filter washes were as described for the colony lifts for the identification of p599 positives. Following autoradiography, probe positive colonies were picked and grown in LB-ampicillin medium. Plasmid preparations from these were subjected to restriction analysis. Correct recombinants, with the HSA minigene cloned into the SnaB I site in the same orientation as the BLG promoter, were identified by the production of a single EcoR I fragment (approximately 9800 bp) and EcoR I/Sal I double digest fragments of approximately 3511, 3419 and 2850 bp and were designated p600.

Example 6

A. CONSTRUCTION OF BLG VECTOR WITH AN HSA MINIGENE CONTAINING HSA INTRONS 1 AND 2 (p607)

In preparing this construct the HSA cDNA was subcloned into pGEM-1. pGEM-1 was digested with Pvu II (just outside of the polylinker) and EcoR I (within the polylinker). The 2819 bp vector fragment was gel and elutip purified and ethanol precipitated. The HSA cDNA subcloned in the plasmid vector pBS$^-$ (pHSA-F1$^-$) as described in the section on the construction of p575, was released from pHSA-F1$^-$ by digestion with Sal I which was blunted with Klenow polymerase and excess dNTPs and subsequently digested with EcoR I. The 2004 bp fragment of the HSA cDNA was gel and elutip purified and ethanol precipitated. This cDNA fragment was ligated into the purified pGEM-1 plasmid fragment digested with Pvu II (blunt) and EcoR I. Ligation products were transformed into DH5 cells and positive transformants selected on LB-ampicillin plates. Selected colonies were lifted onto Whatman 541 filters as previously described. A ^{32}P-labeled HSA cDNA probe was produced by the

random primer technique using a 1941 bp Hind III fragment of pHSA-F1- as a template. Hybridization was in standard buffer at 65°C. Washing conditions included a final, most stringent wash of 0.5 x SSC, 0.2% SDS at 65°C. Probe positive colonies were picked, grown in LB-ampicillin medium. Plasmids obtained from these cultures were subjected to restriction analysis. Correct recombinants were characterized by the presence of 2818 and 2011 bp BamH I fragments and 2806, 1165 and 858 bp Xba I fragments. Correct clones were designated p597.

A DNA fragment encompassing the HSA genomic sequence including part of exon 1, intron 1, exon 2, intron 2 and part of exon 3 was produced by PCR technology using the following synthetic oligonucleotide primers.

Exon 1 Sense Primer

HindIII Met BstEII

5'-GTACATAAGCTTTGGCACAATGAAGTGGGTAACCTT-3'

Exon 1 sequence

Exon 3 Antisense Primer

3' PvuII BamH I

GACTACTCAGTCGACTTTTAACACCTAGGGGACTG-5'

Exon 3 sequence

High molecular weight DNA purified from human lymphocytes was used as a template. This PCR product was digested with Hind III and Pvu II and the resultant 2422 bp fragment gel and elutip purified and ethanol precipitated. This fragment was ligated into a pGEM-1 vector prepared by digestion with Hind III and Pvu II, gel and elutip purification and ethanol precipitation of the resultant 2774 bp fragment. The pGem fragment was subsequently dephosphorylation with CIP. Ligation products were used to transform DH5 cells to ampicillin resistance. Correct recombinants clones were identified by the presence of a BstEII site resulting in linearization of plasmid (5196 bp) and the generation of 2774 and 2422 bp fragments upon digestion with Hind III and Pvu II. Correct recombinants were designated p594.

HSA introns 1 and 2 were introduced into the HSA cDNA as follows. HSA sequences from exon 1 to exon 3 including introns 1 and 2 were released

from p594 by digestion with BstEII (within exon 1) and Pvu II (within exon 3) as a 2401 bp fragment. This fragment was gel and elutip purified and ethanol precipitated. Plasmid p597 was similarly digested with BstEII and Pvu II. The 4596 fragment (which lacks the cDNA sequences between these sites in the cDNA), was gel and elutip purified and ethanol precipitated. These two prepared fragments were ligated and ligation products transformed into DH5 cells to ampicillin resistance. Transformants containing the HSA introns were identified by Whatman 541 filter lifts and hybridization with a ^{32}P-5'-end labeled (using T4 polynucleotide kinase) synthetic oligonucleotide probe. The synthetic oligonucleotide used has HSA intron 2 specific sequence, i.e., 5' GTCACATGTGGCTAATGGCTACTG 3'.

Hybridization was in standard buffer at 60°C. Filters were washed with the final wash of 2 x SSC, 0.5% SDS, 60°C. Positive colonies were subjected to restriction analysis. Correct recombinants were identified by 2 BamH I (approximately 4174 and 2818 bp) fragments and 2 EcoR I (approximately 3765 and 3227 bp) fragments. These correct clones contain an HSA minigene possessing HSA introns 1 and 2 and were designated p603.

The HSA minigene with introns 1 and 2 was introduced into BLG vector p590 as follows. Vector p590 was digested with SnaB I and dephosphorylated with CIP. The HSA minigene was released from p603 as a 4174 bp BamH I fragment which was gel and elutip purified and ethanol precipitated. The BamH I ends of this fragment were blunted using Klenow polymerase and excess dNTPs. The prepared minigene from p603 and the prepared vector, p590, were ligated together. Ligation products were transformed into DH5 cells to ampicillin resistance. Correct clones were identified by the presence of 2 EcoR I fragments (approximately 7485 and 3765 bp) and 2 Xba I fragments (approximately 9196 and 2054 bp). The correct clones have the HSA minigene inserted into the SnaB I site of the BLG vector p590 in the same orientation as the BLG promoter and were designated p607.

B. CONSTRUCTION OF A BLG VECTOR WITH A DOWNSTREAM SV40 POLYADENYLATION POLY A SIGNAL (p589)

In order to examine whether the polyadenylation signal source affected expression of the HSA protein, a BLG vector having the BLG coding sequence

replaced by an HSA coding sequence was constructed having an SV40 polyadenylation signal instead of the BLG poly A signal. The BLG sequences contained within the construct made here are only those upstream of the BLG coding sequence which include the promoter region. The BLG coding sequences as well as downstream sequences were deleted. The construct maintains the SnaB I site in BLG exon I for the insertion of foreign genes, in this case HSA. An SV40 polyadenylation signal was placed downstream of the SnaB I site in order to supply this, 3'-RNA processing signal.

Plasmid p583 containing the 5'-EcoR I subgenomic portion of the BLG gene with an SnaB I site introduced in exon 1 was digested with SnaB I and partially digested with Sal I. The DNA fragment of approximately 5800 bp made up of almost the entire pGEM-1 plasmid digested at its native Sal I site within its polylinker and the 5'-end of the 5'-portion of the BLG gene to the introduced SnaB I site was gel and elutip purified and ethanol precipitated.

The SV40 early gene (T and t) polyadenylation signal poly A was released from SV40 DNA by restriction with BclI at its 5'-end (SV40 map position 2770) and BamH I at its 3'-end (SV40 map position 2533). The 237 bp poly A signal and site fragment was gel and elutip purified and ethanol precipitated. This fragment was ligated into the BamH I site (dephosphorylated with CIP) of pGEM2. Ligation products were transformed into HB101 cells to ampicillin resistance. As the SV40 poly A signal fragment was able to be ligated into the BamH I site of pGEM in either orientation, the desired orientation was determined by analysis of plasmid DNAs from selected clones by digestion with DraI. Clones with the desired orientation, (the SV40 poly A signal fragment 5'-end (BclI end) downstream of the polylinker Xba I and Sal I sites) were characterized by DraI fragments of 1203, 1192, 692 and 19 bp. Desired recombinants were designated p290. Additional restriction sites including SnaB I were introduced upstream of the polyA signal as follows. Plasmid p290 was digested with Sac I and AvaI (within the pGEM2 polylinker), the large fragment gel and elutip purified and ethanol precipitated. Into the resultant fragment was ligated synthetic oligonucleotides which when annealed were of the sequence;

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      Sac I      SnaB I      Hind III
5'       CCTCGAGTACGTAAGATCTAAGCTTC        3'
3'   TCGAGGAGCTCATGCATTCTAGATTCGAAGGGCC    5'
        Xho I       Bgl II      Ava I
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Ligation products were transformed into E. coli HB101 cells to ampicillin resistance. Resultant correct recombinants which now possessed these additional multiple cloning sites upstream of the poly A signal sequence were designated p299.

The SV40 poly A signal and site sequences were released from p299 by digestion at the upstream SnaB I and downstream Sal I sites. This 270 bp fragment was gel and elutip purified and ethanol precipitated. It was ligated into the SnaB I/partial Sal I purified fragment from p583. Ligation products were transformed into DH5 cells to ampicillin resistance. Correct recombinants were identified by linearization by SnaB I (approximately 6200 bp) and the generation of 2 fragments (approximately 3400 and 2800 bp) upon digestion with Sal I. Correct recombinants were designated p589.

C. CONSTRUCTION OF BLG VECTOR (BLG 5'-SEQUENCES, SV40 3'-POLY A SIGNAL, NO BLG CODING SEQUENCE) WITH AN HSA MINIGENE CONTAINING HSA INTRON 1 (p598)

Construct p589 was digested with SnaB I and resultant restricted ends dephosphorylated with CIP. This DNA was ligated with the prepared HSA minigene with intron 1 blunt ended as described in the section on the concentration of p599 and ligation products introduced into DH5 cells by transformation. Correct recombinants with the HSA minigene cloned into the SnaB I site in the same orientation as the BLG promoter were identified by the production of a single EcoR I linear fragment (approximately 8800 bp) and EcoR I/Sal I fragments of approximately 3450, 2850 and 2500 bp. These correct clones were designated p598.

D. INTRODUCTION OF AN HSA MINIGENE CONTAINING THE FIRST HSA INTRON INTO A BLG VECTOR WITH EXTENDED 5'-SEQUENCES (p643, p647)

In order to ascertain whether longer 5' BLG would increase the expression of HSA, an HSA minigene, with intron 1, was introduced into BLG constructs with extended 5'-BLG sequences (5.5 Kb or 10.8 Kb). BLG coding

and 3'-sequences are not included into these vectors. The SV40 sequences containing the polyadenylation signal and site are included.

In order to produce the clone with the BLG 5.5 Kb 5'-sequence construct p640 was digested with Asp 718, (between the original BLG 5'-EcoR I site and the transcriptional initiation site), and Pvu I (within pGEM). The fragment (approximately 6143 bp) which includes pGEM sequences and the BLG sequences upstream of the Asp 718 site was gel and elutip purified and ethanol precipitated. Vector p598 was similarly digested with Asp 718 and Pvu I. The fragment (approximately 5184 bp) which includes pGEM sequences, complementary to those above, as well as BLG 5'-sequences downstream of the Asp 718 site, the HSA minigene with its first intron and the SV40 poly A site, was gel and elutip purified and ethanol precipitated. These two fragments were ligated together and ligation products transformed into DH5 cells to ampicillin resistance. Correct recombinants were identified by the generation of 3 fragments (approximately 8164, 2831 and 320 bp) upon Hind III digestion and were designated p643.

The BLG 10.8 Kb 5'-sequence construct was made in a similar manner as for p643. Plasmid p645 was digested with Asp 718 and Pvu I. The resultant fragment (approximately 11,384 bp) was gel and elutip purified and ethanol precipitated. This fragment was ligated to the purified fragment (approximately 5184 bp) generated by the digestion of p598 with Asp 718 and Pvu I discussed above. Ligation products were transformed into DH5 cells to ampicillin resistance. Correct recombinants were identified by the generation of 5 DNA fragments (approximately 8159, 3460, 2829, 1800 and 320 bp) upon digestion with Hind III. Correct recombinants were designated p647 and include 5'-BLG sequences of 10.8 Kb in combination with an HSA minigene possessing intron 1.

Example 7

CONSTRUCTION OF BLG VECTORS WITH AN HSA MINIGENE CONTAINING HSA INTRONS 1-6 (p652, p661)

Transgenic BLG constructs with an HSA minigene containing HSA introns 1-6 were made by taking advantage of the unique BstEII (within exon 1) and the Nco I (within exon 7) sites within the HSA gene.

Construct p598, which is equivalent to the desired vector except that its HSA minigene contains only intron 1, was digested with BstEII and Nco I. The DNA fragment (approximately 7304 bp) lacking HSA sequences between these two sites was gel and elutip purified and ethanol precipitated. Plasmid p650 was similarly digested with BstEII and Nco I. The resultant 7,756 bp fragment containing the HSA gene region between these sites including introns 1-6 was also gel purified and ethanol precipitated. The two purified fragments were ligated together and transformed into TG1 cells to ampicillin resistance. Correct recombinants were identified by 4 DNA fragments (approximately 9819, 4682, 320, and 240 bp) upon digestion with Hind III and 5 fragments (approximately 9552, 1882, 1296, 1258, and 1073 bp) upon digestion with Xba I. These were designated p652 (ATCC No. 68653). Construct p652 possesses a SV40 poly A site.

A second BLG vector similar to p652, except that 3'-sequences containing the polyadenylation signal and site were contributed by BLG 3'-sequences, was constructed in a parallel manner. Construct p600 was digested with BstEII and Nco I. The DNA fragment of approximately 8266 bp, lacking HSA sequences between these sites was gel and elutip purified and ethanol precipitated. It was ligated with the purified 7756 bp p650 BstEII - Nco I fragment discussed above, composed of HSA gene sequences between these two sites. Ligation products were transformed into DH5 cells to ampicillin resistance. Correct recombinants were identified by the possession of unique BstEII and Nco I sites, resulting in the generation of a single DNA fragment (approximately 16021 bp) upon digestion with each restriction enzyme. In addition, correct clones were identified by the generation of 3 DNA fragments (10945, 4171, and 905 bp) upon digestion with BamH I. Correct clones were designated p661.

Example 8

CONSTRUCTION OF BLG VECTOR WITH AN HSA MINIGENE CONTAINING HSA INTRONS 7-14 (p687)

This transgenic vector made up of an HSA minigene with introns 7-14 under the control of the BLG promoter and with an SV40 poly A site was constructed in several steps. The downstream HSA gene region containing introns 12-14 (within p651) was combined with HSA cDNA sequences up to intron 12, thereby creating an HSA minigene with introns 12-14. An SV40 poly A site was then joined to the 3'-end of this minigene. This was then manipulated to include introns 7-11 to produce an HSA minigene with introns 7-14, with an SV40 poly A site. Finally, the minigene was recombined with the 5'-BLG promoter sequences resulting in construct p687.

In the first step, construct p651 was digested with Asp I (within HSA exon 12) and Hind III (within HSA intron 15). The resultant, desired, fragment (2972 bp) extending from exon 12 to exon 15 was gel and elutip purified. Translational termination of the HSA RNA occurs within exon 14 derived sequences and polyadenylation of the HSA transcript occurs downstream of the Hind III site. Therefore, the isolated fragment has been separated from the HSA poly A signal and site but has not had any coding sequences deleted. Construct p597 (HSA cDNA within pGEM-1) was similarly digested with Asp I and Hind III. The resultant fragment (approximately 4263 bp) with the HSA cDNA sequences between the Asp I (within exon 12 derived sequences) and Hind III (within exon 15 derived sequences) was gel and elutip purified. The two fragments were ligated together and ligation products introduced into E. coli MC1061 cells by electroporation to ampicillin resistance. The correct recombinants were characterized by the presence of single Asp I and Hind III sites as well as 2 fragments (approximately 4275 and 2800 bp) upon digestion with EcoR I and BamH I and were designated p668. Construct p668 contains an HSA minigene with introns 12-14 within pGEM-1.

SV40 poly A signal and site sequences were introduced downstream of the HSA minigene in p668. Construct p668 was digested with Hind III (at the downstream end of HSA sequences) and the blunt cutter Nae I (within pGEM sequences approximately 300 bp from the Hind III site). The fragment (approximately 6800 bp), deleted of the 300 bp between the two sites, was gel and elutip purified. SV40 poly A site sequences were released from p299 by first digesting with PstI (within the multiple cloning site adjacent to the downstream end of the poly A site sequences). The Sal I site between the poly

A site sequences and the PstI site remains adjacent to the downstream end of the poly A site sequences. The digested PstI site was blunted by filling in with T4 DNA polymerase in the presence of excess dNTPs. The DNA was then digested with Hind III (within the multiple cloning site adjacent to the upstream end of the poly A site sequences) and the released SV40 sequences fragment (approximately 200 bp) was gel and elutip purified. The two purified DNAs were ligated together and transformed into E.coli DH5 cells to ampicillin resistance. Correct clones with the SV40 poly A site ligated at the downstream end of the HSA minigene in the same orientation were characterized by the generation of 2 fragments (approximately 4700 and 2300 bp) upon Sal I digestion and 3 fragments (approximately 4200, 2300 and 500 bp) upon Sal I and EcoR I digestion. These correct constructs were designated p674.

The introduction of HSA introns 7-11 into p674 containing the HSA minigene with introns 12-14 was accomplished by a tripartite ligation. The HSA gene sequences contained within p679 were released by digestion with Nco I (within exon 7) and Hind III (within the downstream end of intron 8) as a fragment of 2814 bp which was gel and elutip purified. The HSA sequences contained within p676 were released by digestion with Hind III (the same site within the downstream end of intron 8 described for p679) and partial digestion with Asp I (within exon 12; a second Asp I site is found within intron 11) as a fragment of 3237 bp which was gel and elutip purified. Construct p674 (containing the HSA minigene with introns 12-14) was digested with Nco I (again within exon 7) and Asp I (again within exon 12). The resultant DNA (approximately 6400 bp) deleted of HSA cDNA sequences between the Nco I and Asp I sites was gel and elutip purified. The 3 purified DNAs were ligated together and ligation products introduced into E.coli MC1061 cells by electroporation to ampicillin resistance. Correct recombinants were characterized by the generation of 2 Asp I fragments (approximately 12406 and 273 bp) and 5 Xba I fragments (approximately 3537, 2719, 2623, 2566 and 1234 bp). These correct constructs, designated p683, are HSA minigenes with introns 7-14, with an adjacent downstream SV40 poly A site within pGEM-1.

Finally, the HSA minigene with introns 7-14 was introduced into a transgenic construct downstream of the BLG 5'-flanking promoter sequences. Construct p683 was digested with Nco I (within HSA exon 7) and Pvu I (within pGEM). The DNA fragment (approximately 10400 bp) made up of the HSA

gene sequences downstream of the Nco I site (including introns 7-14), the SV40 poly A site and the adjacent pGEM sequences was gel and elutip purified. Construct p572 (containing the HSA cDNA within the Pvu II site of BLG exon 1) was similarly digested with Nco I (within exon 7 derived sequences) and Pvu I (within pGEM). The DNA fragment (approximately 5570 bp) made up of pGEM sequences complementary to the pGEM sequences in the fragment described above, BLG 5'-flanking promoter sequences and the HSA cDNA up to the Nco I site within exon 7, was gel and elutip purified. The two purified fragments were ligated together and ligation products were introduced into E.coli DH10B cell by electroporation to ampicillin resistance.

Correct recombinants were characterized by 3 fragments (approximately 10098, 4183 and 1699 bp) generated by digestion with BamH I. These correct constructs, designated p687, contain the HSA minigene with introns 7-14, a downstream SV40 poly A site and an upstream BLG promoter and represent a final BLG/HSA construct for introduction into transgenic animals.

Example 9

CONSTRUCTION OF BLG VECTOR WITH AN HSA MINIGENE CONTAINING INTRONS 2 AND 7-14 (p696)

The construction of this transgenic vector containing an HSA minigene with introns 2 and 7-14 was performed in several steps. The Cla I site which had previously been introduced into HSA exon 2 derived sequences (without altering the encoded amino acid) in an HSA cDNA in plasmid pBS$^-$ (p595) was transferred into an HSA cDNA within a pGEM plasmid. A PCR product extending from HSA exon 2 through intron 2 and into exon 3 was introduced into the homologous region in the HSA cDNA within pGEM using this Cla I site. Finally, intron 2 was transferred into the transgenic construct carrying an HSA minigene with introns 7-14 (p687).

The Cla I site which had been introduced into HSA exon 2 was transferred from the HSA cDNA in construct p595 (pBS$^-$ plasmid) to an HSA cDNA in a pGEM plasmid (p597) because there are multiple Pvu II sites within p595 which interfere with the subsequent recombination strategy (p597 has a unique Pvu II site). Construct p597 was digested with BstEII (within HSA exon

1 derived sequences) and Nco I (within HSA exon 7 derived sequences). The large DNA fragment deleted of the HSA cDNA sequences between the BstEII and Nco I sites was gel and elutip purified. Construct p595 was similarly digested with BstEII and Nco I and the DNA fragment (801 bp) of the HSA cDNA sequences between these sites, including the previously introduced Cla I site, was gel and elutip purified. These two purified DNAs were ligated together and ligation products introduced into E.coli DH10B cells by electroporation to ampicillin resistance. Correct recombinants were characterized by the presence of a Cla I site and were designated p689.

HSA intron 2, with flanking exon regions including the introduced Cla I site in exon 2, was obtained by PCR technology. The HSA exon 2 sense synthetic oligonucleotide primer (with incorporated Cla I site) and the HSA exon 3 antisense primer (with incorporated Pvu II site), shown below, were used for a PCR with construct p594 (containing HSA intron 2 and flanking exons) as template.

HSA Exon 2 5' GGTTGCTCATCGATTTAAAGATTTGGG 3'
Sense Primer Cla I

HSA Exon 3 3' GACTACTCAGTCGACTTTTAAcacttaagggactg 5'
Antisense Primer Pvu II (Non-HSA Sequences)

The reaction was of 28 cycles of 94° C (1'30"), 55° C (2') and 72° C (3'). Following two chloroform extractions and ethanol precipitation the PCR products were digested with Cla I and Pvu II. The resultant 1602 bp DNA was gel and elutip purified. Construct p689 was digested with Cla I (within HSA exon 2) and Pvu II (within HSA exon 3) and the large DNA fragment deleted of HSA cDNA sequences between the Cla I and Pvu II sites was gel and elutip purified. The two purified DNAs were ligated together and ligation products transformed into E.coli DH5 cells to ampicillin resistance. The generation of linear DNA of approximately 6500 bp by Cla I and Pvu II, individually, identified correct recombinants. These were designated p690 and are composed of an HSA minigene with intron 2 within a pGEM plasmid.

Finally, HSA intron 2 was transferred into transgenic vector p687 containing an HSA minigene with introns 7-14. Construct p687 was digested with BstEII (within HSA exon 1) and Nco I (within HSA exon 7). The large DNA fragment deleted of HSA cDNA sequences between the BstEII and Nco I sites was gel and elutip purified. Construct p690 was similarly digested with BstEII and Nco I. The released 2255 bp fragment made up of HSA cDNA from the BstEII site to the Nco I site and includes intron 2, was gel and elutip purified. The two purified DNAs were ligated together and ligation products transformed into E.coli DH5 cells. Correct recombinants were characterized by the release of a 2255 bp fragment upon digestion with BstEII and Nco I. These constructs, designated p696 (ATCC No. 68654), are made up of an HSA minigene with introns 2 and 7-14 under the control of the BLG promoter and flanked downstream by an SV40 poly A site and are suitable for the production of transgenic animals.

Example 10

CONSTRUCTION OF BLG VECTOR WITH A COMPLETE HSA GENE INCLUDING ALL INTRONS, 1-14 (p686)

The construction of the transgenic vector utilizing the entire HSA gene including all 14 of its introns was by recombining construct p652, containing an HSA minigene with introns 1-6, and construct p683, containing an HSA minigene with introns 7-14. Construct p652 was digested with Nco I (within HSA exon 7) and Pvu I (within pGEM). The DNA fragment (approximately 12530 bp) made up of pGEM sequences, the BLG 5'-flanking promoter and the HSA minigene (including introns 1-6) up to the Nco I site in exon 7 was gel and elutip purified. Construct p683 was similarly digested with Nco I (within HSA exon 7) and Pvu I (within pGEM). The DNA fragment (approximately 10400 bp) made up of the HSA minigene downstream of the Nco I site in exon 7 (including introns 7-14), the adjacent downstream SV40 poly A site and its adjacent pGEM sequences complementary to the pGEM sequences in the fragment described above, was gel and elutip purified. The two purified fragments were ligated together and ligation products were introduced into E.coli DH10B cells by electroporation to ampicillin resistance. Correct recombinants were characterized by the generation of two fragments

(approximately 18728 and 4186 bp) upon digestion with BamH I and were designated p686.

All exons were sequenced in this vector and found to be correct thereby confirming that clonings of the HSA gene had been correct.

Example 11

A. CONSTRUCTION OF A VARIANT HSA cDNA WHICH ENCODES A PHE AMINO ACID AT HSA PROTEIN POSITION #403 INSTEAD OF A LEU AMINO ACID (p822)

The HSA cDNA sequence used in the constructs described above encoded an HSA protein sequence which differed from the HSA sequence described by Minghetti et al. (1986) by one amino acid. The Minghetti sequence encodes a Phe amino acid (TTC codon) at position #403 (based upon numbering of amino acids with the first amino acid of the mature HSA protein designated position #1) while that described in the examples above encoded a Leu amino acid (codon CTC). The sequence of Minghetti was prepared in order to demonstrate that the vectors of the present invention were able to result in high level expression of variant forms of HSA. In vitro mutagenesis (site directed mutagenesis) was performed using a commercial kit (Amersham SDM Kit), with single stranded DNA template derived from pHSA-F1$^-$, a synthetic oligonucleotide of the sequence 5'-GGAGAGTACAAATTCCAGAATGCGCTA-3' as a primer for first strand synthesis and a synthetic oligonucleotide of the sequence 5'-AGCGCATTCTGGAATTTGTACTCT-3' as a primer for second strand synthesis. The resultant clone, made up of an HSA cDNA encoding a Phe (TTC codon) at HSA amino acid position #403 within the pBS$^-$ vector, was designated p822. The desired change was verified by DNA sequencing.

B. CONSTRUCTION OF AN IN VITRO ANALYSIS VECTOR FROM p822 (p823)

An in vitro analysis vector containing the HSA cDNA with the Minghetti codon sequence for HSA amino acid #403 was constructed. Construct p822 was digested with Nco I and Avr II (sites which flank the codon for amino acid

#403). The resultant 549 bp DNA fragment, which contained the codon for amino acid #403, was gel and elutip purified. In vitro analysis vector p658 (containing the HSA cDNA) was similarly digested with Nco I and Avr II. The large DNA fragment lacking the HSA sequences between the two sites was gel and elutip purified. The two purified fragments were ligated together and introduced into E. coli DH5 cells to confer ampicillin resistance. Correct recombinants were identified by the generation of a 549 bp fragment upon digestion with Nco I and Avr II. Sequencing verified that the Minghetti codon for amino acid #403 was present. This new in vitro analysis vector was designated p823.

In vitro analysis, as previously described, demonstrated that p823 supports the expression and secretion of immunoprecipitable HSA from transfected mammalian cell line COS-7 cells.

Example 12

A. CONSTRUCTION OF A BLG VECTOR WITH AN HSA MINIGENE CONTAINING INTRONS 1-6 WITH A PHE CODON FOR HSA AMINO ACID #403 (p825)

A transgenic vector containing an HSA minigene with introns 1-6 and encoding the Minghetti amino acid sequence at position #403 was constructed. Vector p652 was digested with Nco I (within HSA exon 7) and Pvu I (within pGEM). The large DNA fragment, comprising the BLG promoter and HSA sequences upstream of the Nco I site, including HSA introns 1-6, was gel and elutip purified. Construct p823 was similarly digested with Nco I and Pvu I. The smaller DNA fragment (approximately 2548 bp) comprising HSA cDNA sequences downstream of the Nco I site (including Minghetti codon sequence for amino acid #403) and the SV40 polyadenylation site was gel and elutip purified. The two purified fragments were ligated together and introduced into E. coli DH5 cells to confer ampicillin resistance. Correct recombinants were identified by the generation of 5 DNA fragments (approximately 9308, 1882, 1296, 1257 and 1091 bp) upon digestion with Xba I and 6 DNA fragments (approximately 5916, 3338, 2137, 1768, 1039 and 636 bp) upon digestion with Asp I. This new transgenic vector was designated p825.

B. CONSTRUCTION OF AN IN VITRO ANALYSIS VECTOR FROM p825 (p829)

An in vitro analysis vector was made from transgenic vector p825 (possessing an HSA minigene with introns 1-6, Minghetti codon for amino acid #403) by introducing an SV40 enhancer into the Asp 718 site within the BLG promoter as previously described for the construction of in vitro vector p656 from transgenic vector p652. This new vector was designated p829.

In vitro analysis , as previously described, demonstrated that ivector p829 supports the expression and secretion of immunoprecipitable HSA from transfected mammalian cell line COS-7 cells.

Example 13

CONSTRUCTION OF BLG VECTORS WITH AN HSA MINIGENE CONTAINING INTRONS12-14 WITH EITHER LEU OR PHE CODON FOR HSA AMINO ACID #403 (p688, p824)

A transgenic construct containing an HSA minigene with introns 12-14 was made as follows: Construct p674 was digested with Nco I (within HSA exon 7) and Pvu I (within pGEM). The large DNA fragment generated, comprised of the HSA sequences downstream of the Nco I site, including HSA introns 12-14, and the SV40 polyadenylation site was gel and elutip purified. Construct p572 was similarly digested with Nco I and Pvu I and the large fragment generated comprised of the BLG promoter and HSA sequences (cDNA) upstream of the Nco I site was gel and elutip purified. The two purified fragments were ligated together and introduced into E. coli DH10B cells to confer ampicillin resistance. Correct recombinants were identified by the generation of 3 DNA fragments (approximately 4767, 4177 and 1700 bp) upon digestion with BamH I and 3 DNA fragments (approximately 7028, 2757 and 859 bp) upon digestion with Xba I. This new transgenic vector possessing an HSA minigene with introns 12-14 was designated p688.

Transgenic vector p688 containing an HSA minigene with introns 12-14 was modified so that it encodes the Minghetti amino acid sequence at position

#403. The 549 bp Nco I to Avr II DNA fragment obtained from construct p822 was used to replace the corresponding fragment in construct p688 (similarly digested with Nco I and Avr II) as previously described for the construction of vector p823. This new transgenic construct was designated p824.

Example 14

A. CONSTRUCTION OF BLG VECTORS WITH AN HSA MINIGENE CONTAINING INTRONS 1 and 2 and 12-14 WITH EITHER LEU OR PHE CODON FOR HSA AMINO ACID #403 (p812, p826)

A transgenic construct containing an HSA minigene with introns 1+2+12-14 was made as follows: Construct p674 was digested with Nco I (within HSA exon 7) and Pvu I (within pGEM). The large DNA fragment generated, comprised of the HSA sequences downstream of the Nco I site, including HSA introns 12-14, and the SV40 polyadenylation site was gel and elutip purified. Construct p607 was similarly digested with Nco I and Pvu I and the large fragment generated comprised of the BLG promoter and HSA sequences upstream of the Nco I site, including HSA introns 1 and 2, was gel and elutip purified. The two purified fragments were ligated together and introduced into E. coli DH5 cells to confer ampicillin resistance. Correct recombinants were identified by the generation of 3 DNA fragments (approximately 8900, 2718 and 854 bp) upon digestion with Xba I. This new transgenic vector possessing an HSA minigene with introns 1+2+12-14 was designated p812.

A transgenic construct containing an HSA minigene with introns 1+2+12-14 with the Minghetti codon for HSA amino acid #403 was made. Construct p824 was digested with Nco I (within HSA exon 7) and Pvu I (within pGEM). The large DNA fragment generated, comprised of the HSA sequences downstream of the Nco I site (including HSA introns 12-14 and Minghetti codon for #403) and the SV40 polyadenylation site was gel and elutip purified. Construct p607 was similarly digested with Nco I and Pvu I and the large fragment generated comprised of the BLG promoter and HSA sequences upstream of the Nco I site, including HSA introns 1 and 2, was gel and elutip purified. The two purified fragments were ligated together and introduced into E. coli DH5 cells to confer ampicillin resistance. Correct recombinants were

identified by the generation of 3 DNA fragments (approximately 8900, 2718 and 854 bp) upon digestion with Xba I. This new transgenic vector was designated p826.

B. CONSTRUCTION OF IN VITRO ANALYSIS VECTORS FROM p812 AND p826 (p813, p830)

An in vitro analysis vector was made from transgenic vector p812 (possessing an HSA minigene with introns 1+2+12-14) by introducing an SV40 enhancer into the Asp 718 site within the BLG promoter as previously described for the construction of in vitro vector p656 from transgenic vector p652. This new vector was designated p813.

In vitro analysis , as previously described, demonstrated that vector p813 supported the expression and secretion of immunoprecipitable HSA from transfected mammalian cell line COS-7 cells.

An in vitro analysis vector was made from transgenic vector p826 (possessing an HSA minigene with introns 1+2+12-14, Minghetti codon for amino acid #403) by introducing an SV40 enhancer into the Asp 718 site within the BLG promoter as previously described for the construction of in vitro vector p656 from transgenic vector p652. This new in vitro analysis vector was designated p830.

In vitro analysis , as previously described, demonstrated that vector p830 supports the expression and secretion of immunoprecipitable HSA from transfected mammalian cell line COS-7 cells.

Example 15

IN VITRO (TISSUE CULTURE) ANALYSIS OF BLG/HSA VECTORS

In order to determine that all of the BLG/HSA vectors introduced into transgenic animals had the potential ability to support the expression of HSA in the milk of such animals, the ability to support expression of HSA in tissue culture cells was first tested. The natural in vivo regulation of expression of milk proteins under the control of their native promoters (e.g.,. BLG) is complex

and requires the influence of hormones and specific cell-cell interactions. The BLG 5'-flanking promoter sequences are not usually active in tissue culture cells and tissue culture systems which precisely mimic the natural in vivo conditions did not exist.

In order to stimulate these sequences into activity an SV40 enhancer was introduced within the promoter. This allowed the testing of the levels of expression of HSA in tissue culture supported by BLG/HSA constructs which differ in their HSA gene makeup (cDNA, minigenes, gene). In order to keep the genetic background the same in these in vitro analysis constructs, a series of constructs were made which differed only in the HSA gene components. An SV40 enhancer was first introduced into transgenic construct p652 (3 kb BLG 5'-flanking promoter sequences; HSA minigene with introns 1-6; SV40 poly A site). The resultant in vitro construct was then used to make all other constructs of this series so that while the HSA sequences varied, the BLG promoter with introduced SV40 enhancer was identical in all. In addition all possessed the same SV40 poly A site downstream of the HSA sequences.

Construct p652 was digested with Asp 718 (within the BLG promoter, approximately 900 bp upstream of the BLG transcriptional start site). The linearized construct was extracted two times with phenol/chloroform and ethanol precipitated. The Asp 718 digested ends were blunted by filling in with Klenow enzyme (Boehringer Mannheim) in the presence of excess dNTPs. Following the fill in reaction, the enzyme was heat inactivated (65° C, 15') in the presence of 10 mM EDTA. The sample was again extracted two times and ethanol precipitated. The linearized and blunted DNA was gel and elutip purified and it's 5'-ends dephosphorylated with calf intestinal alkaline phosphatase (Promega). The enzyme was heat inactivated (65° C, 15') in the presence of 20 mM EGTA. The sample was again extracted two times and ethanol precipitated. The SV40 enhancer was released from construct pSV_2CAT (Gorman et al., 1982, Mol. Cell. Biol. 2, 1044-1051) as a 179 bp Fok I (cleaves at SV40 bp position 94) to Pvu II (cleaves at SV40 bp position 273) fragment. The Fok I site was blunt ended with Klenow polymerase and excess dNTPs. The fragment was gel and elutip purified and subsequently ligated to the prepared p652 fragment. Ligation products were transformed into E.coli TG1 cells to ampicillin resistance. Correct recombinants were identified by digestion with BamH I and EcoR I whose sites flank the Asp 718 site of p652.

The conversion of the approximate 2088 bp fragment to approximately 2267 bp identified correct recombinants with an SV40 enhancer introduced into the Asp 718 site of p652. These were designated p656. (Fig. 2C) In vitro analysis construct, p656 is capable of expression of the HSA minigene with introns 1-6 in tissue culture cells.

Several new in vitro analysis constructs were made directly using in vitro analysis construct p656. Construct p656 was digested with BstEII (within HSA exon 1) and Nco I (within HSA exon 7). The large DNA fragment deleted of HSA sequences between these two sites was gel and elutip purified. DNA fragments of HSA sequences between the BstEII site in exon 1 and the Nco I site in exon 7 made up of cDNA (801 bp) or which included intron 1 (1510 bp) or intron 2 (2255 bp) or introns 1 and 2 (2964 bp) were derived from p582 (containing HSA cDNA, discussed below), p600 (containing HSA minigene with intron 1), p690 (containing HSA minigene with intron 2) or p607 (containing HSA minigene with introns 1 and 2), respectively, by digestion with BstEII and Nco I. Fragments were gel and elutip purified and individually ligated into the purified p656 fragment lacking sequences between these two sites. Ligation products were introduced into E.coli DH5 alpha cells by transformation or E.coli DH10B cells by electroporation to ampicillin resistance. Correct recombinants were identified by the generation of DNA fragments of 801, or 1510, or 2255, or 2964 bp, respectively, upon digestion with BstEII and Nco I. The new in vitro analysis construct containing HSA cDNA was designated p658. The constructs containing HSA minigene with intron 1 was designated p659, with intron 2, p691, and with introns 1 and 2, p660.

In addition to making a BLG/HSA in vitro analysis construct with the HSA cDNA, we also made an in vitro analysis construct with the HSA cDNA under the control of the highly active Adenovirus major late promoter and SV40 enhancer combination. This allowed the evaluation of HSA expression from its cDNA in a construct other than a BLG construct. This construction was made in two steps. In the first step the SV40 early region small t splicing signals and poly A site was placed downstream of a polylinker, which itself is downstream of the major late promoter. An in vitro analysis construct (referred to here as p550) made up of the major late promoter with an SV40 enhancer introduced at its EcoRV site and followed by a polylinker (Hurwitz et al., 1987, Nucl. Acids Res. 15, 7137-7153.) was digested with BamH I (within the polylinker). The

linearized DNA (2818 bp) was gel and elutip purified. It's 5'-ends were dephosphorylated with calf intestinal alkaline phosphatase. The enzyme was heat inactivated as previously described, extracted with phenol/chloroform two times and ethanol precipitated. This fragment was ligated to an approximately 850 bp fragment (Bgl II at the upstream end and BamH I at the downstream end) consisting of SV40 small t splicing signals and a downstream poly A site (Mulligan and Berg, Science 209:1423-1427, 1980). Ligation products were introduced into E.coli DH5 cells by transformation to ampicillin resistance. Correct recombinants with the splicing signals and poly A site within the BamH I site of p550 in the same orientation as the major late promoter were characterized by the generation of two fragments (approximately 2809 and 856 bp) upon digestion with BamH I and EcoR I. These were designated p566. The HSA cDNA was then introduced into p566. Construct p566 was digested with the blunt cutter Nae I and EcoR I just downstream of Nae I (both sites are within the polylinker between the major late promoter and the downstream SV40 splicing signals and poly A site). The HSA cDNA was obtained as follows. Construct pHSA-F1$^-$ was digested with BamH I (at the 5'-end of the HSA cDNA) and ethanol precipitated. The digested BamH I site was blunted with Klenow enzyme in the presence of excess dNTPs. The DNA was ethanol precipitated and digested with EcoR I (at the 3'-end of the HSA cDNA). The resultant cDNA fragment (1983 bp) was gel and elutip purified. It was then ligated into the prepared p566 DNA and ligation products introduced into DH5 cells by transformation to ampicillin resistance. Correct recombinants were characterized by the restoration of the unique EcoR I site and the generation of fragments (approximately 4208, 1399 and 36 bp) upon digestion with Bgl II. This in vitro analysis construct with the HSA cDNA under the control of the major late promoter was designated p582.

HSA introns 12-14 were introduced into p658 as follows. Construct p658 was digested with Nco I (within HSA exon 7) and partially with Sal I (at the downstream end of the SV40 poly A site; a second Sal I site is found at the upstream end of the BLG promoter). The DNA fragment of approximately 6000 bp deleted of HSA sequences downstream of the Nco I site in exon 7 and the SV40 poly A site was gel and elutip purified. Construct p674 was digested with Nco I (within HSA exon 7) and Sal I (at the downstream end of the adjacent SV40 poly A site). The DNA fragment of approximately 4000 bp made up of HSA sequences downstream of the Nco I site in exon 7, including exons 12-14,

and the poly A site was gel and elutip purified. These two fragments were ligated together and products were introduced into E.coli MC1061 cells by electroporation to ampicillin resistance. Correct recombinants were characterized by 2 DNA fragments (approximately 8021 and 2824 bp) upon digestion with Sal I and 3 fragments (approximately 7229, 2757 and 859 bp) upon digestion with Xba I. This new in vitro analysis construct, designated p682, contains an HSA minigene with introns 12-14.

A construct containing an HSA minigene with introns 7-14 was made by first digesting p658 (containing an HSA cDNA) with Nco I (within HSA exon 7) and Pvu I (within pGEM). The large DNA fragment (approximately 5569 bp) made up of the BLG promoter with introduced SV40 enhancer and HSA cDNA to the Nco I site in exon 7, as well as pGEM sequences to the Pvu I site adjacent to BLG sequences was gel and elutip purified. Construct p683 was also digested with Nco I (within HSA exon 7) and Pvu I (within pGEM). The DNA fragment (approximately 10400 bp) made up of HSA sequences downstream of the Nco I site, including introns 7-14, the SV40 poly A site and adjacent pGEM sequences (complementary to those found in the fragment above) was gel and elutip purified. The two purified fragments were ligated together and ligation products were introduced into E.coli DH5 alpha cells by electroporation to ampicillin resistance. Correct recombinants were identified by the generation of 2 fragments each upon digestion with BamH I (approximately 11998 and 4185 bp) or Hind III (approximately 9973 and 6210 bp). This new in vitro analysis construct with an HSA minigene with introns 7-14 was designated p684.

HSA introns 1 or 2 or 1 and 2 were introduced into the new in vitro analysis constructs already containing HSA minigenes with either introns 12-14 (p682) or introns 7-14 (p684). Constructs p682 and p684 were each digested with BstEII (within HSA exon 1) and Nco I (within HSA exon 7) and the resultant large DNA fragments deleted of HSA sequences between these two sites were gel and elutip purified. Into each were individually ligated the purified DNA fragments, discussed above, (1510, or 2255 or 2964 bp) of HSA sequences between the BstEII and Nco I sites including intron 1 or intron 2 or introns 1 and 2, respectively. Ligation products were either introduced into E.coli DH5 cells by transformation or E.coli DH10B cells by electroporation. Correct recombinants were identified for each set of parental clones, p682 and

p684, by the generation of 1510 bp (for the introduction of intron 1) 2255 bp (for the introduction of intron 2) or 2964 bp (for the introduction of introns 1 and 2) DNA fragments upon digestion with BstEII and Nco I. The designations of the new in vitro analysis constructs and their specific HSA minigene structure is listed below.

p694	HSA minigene with introns	1 + 12 - 14
p695	" " " "	2 + 12 - 14
p697	" " " "	1 + 2 + 12 - 14
p693	" " " "	1 + 7 - 14
p692	" " " "	2 + 7 - 14
p698	" " " "	1 + 2 + 7 - 14

An in vitro analysis construct containing the entire HSA gene with all 14 introns was made as follows. Construct p656 was digested with Nco I (within HSA exon 7) and Pvu I (within pGEM). The DNA fragment (approximately 12326 bp) made up of pGEM sequences (from the Pvu I site), adjacent BLG promoter with introduced SV40 enhancer and the HSA sequences to the Nco I site within exon 7 including introns 1-6 was gel and elutip purified. To this fragment was ligated the purified p683 Nco I to Pvu I fragment (approximately 10400 bp), discussed above, made up of HSA sequences downstream of the Nco I site in exon 7, including introns 7-14, the SV40 poly A site and adjacent pGEM sequences to the Pvu I site. Ligation products were introduced into E.coli DH10B cells by electroporation to ampicillin resistance. Correct recombinants were characterized by the generation of two fragments (approximately 18929 and 4186 bp) upon digestion with BamH I. This in vitro analysis construct containing the entire HSA gene with introns 1-14 was designated p685.

The in vitro tissue culture expression was accomplished by transient transfection. Tissue culture mammalian cell line COS-7 cells were split equally into 100 mm tissue culture dishes in DMEM medium plus 10% fetal calf serum (FCS) so that they were approximately 50-75% confluent (approximately $5x10^6$ cells). They were incubated overnight at 37 °C in a CO_2 incubator. The next morning the medium was replaced with 5 ml of fresh medium and cells incubated for 1-2 hours. They were then transfected with BLG/HSA constructs (which included the SV40 enhancer) using the calcium phosphate technique (reagents supplied by 5 Prime → 3 Prime, Inc.) by the supplier's protocol. Within each experiment the total amount of the largest construct (kb), for that

experiment, transfected into cells within a plate was 25 µg . In order to transfect equal molar amounts of smaller constructs, the amounts of each of these constructs were reduced proportionally to their size differences to the largest construct and the total amount of DNA for the each construct was brought up to 25 µg using high molecular weight (HMW) salmon sperm (ss) DNA. Following transfection of cells for 4-5 hours, cells were glycerol shocked (3 ml, 2 minutes) and washed according to supplier's protocol and subsequently incubated in 10 or 15 ml of DMEM medium plus 10% FCS for 3 days.

In order to detect the transient expression and secretion of HSA, transfected cells were starved for amino acids cysteine (Cys) and methionine (Met) by first washing with and then incubating cells in DMEM medium (plus glutamine) lacking Cys and Met plus 5% dialyzed FCS (dFCS) for 1-3 hours. Following removal of medium from cells, cells (and de novo synthesized proteins) were metabolically labeled with 3 ml DMEM (plus glutamine, without Cys or Met, plus 10% dialyzed FCS) containing ^{35}S-Cys and ^{35}S-Met (Expre^{35}S^{35}S ^{35}S-Protein labeling mix; New England Nuclear, Inc.) at approximately 200 uCi/ml for 4-5 hours.

After metabolic labeling, the supernatants were harvested from dishes and centrifuged to remove any contaminating cells. Metabolically labeled HSA expressed and secreted into supernatants was detected by immuno-precipitation using rabbit anti-HSA antibodies (DAKO - immunoglobulins Cat. #A001). Supernatants were first precleared with 200 ul of 50% slurry of protein A-Sepharose beads in immunoprecipitation (IPP) buffer (20 mM Tris, pH 8, 150 mM NaCl, 1% NP-40, 0.1% SDS, 2 µg /ml aprotinin) at 4 °C for 30-60 minutes with rocking. Cleared supernatants were separated from beads by centrifugation and treated with rabbit anti-HSA IgG prebound to protein A-Sepharose beads, at 4 °C for 3-4 hours. Beads were washed 6 times with cold IPP buffer, resuspended in 2 X SDS-PAGE Laemmli sample buffer, heated to 95 °C for 5 minutes and run on 8% SDS-PAGE gels. Following electrophoresis, gels were fixed (10% acetic acid, 25% isopropanol), treated with the fluorographic reagent Amplify (Amersham, Inc.), dried onto Whatman 3MM paper and used to expose X-ray film. Developed films (autoradiographs) allowed visualization of the relative levels of expression and secretion of metabolically labeled HSA from each of the tissue culture transient assay plates supported by each of the analyzed BLG/HSA constructs.

The first in vitro analysis demonstrated that HSA can be expressed from constructs containing the HSA cDNA within a BLG construct containing the BLG coding as well as BLG 3'-sequences and polyadenylation poly A site (p615), an HSA minigene containing intron 1 within constructs lacking the BLG coding sequences but which possess either the BLG poly A site (p606), or the SV40 poly A site (p608), or an HSA minigene with introns 1 and 2 within a construct with the BLG poly A site (p610). The HSA produced from these vectors comigrate with HSA produced from a non-BLG transient vector with the HSA cDNA under the control of an SV40 enhancer/Adenovirus major late promoter (p582). As expected BLG constructs which lack the SV40 enhancer (p600) do not express HSA in vitro. The immunoprecipitation of the band seen in this in vitro analysis is specific to the anti-HSA serum and is not precipitated with a non-specific antiserum demonstrating that the band is in fact HSA. Significantly, the levels of expression increase with the increase in number of introns with the cDNA being expressed least and the HSA minigene with introns 1 and 2 expressed to the highest level in this group. The levels of expression from p606 and p608 (HSA minigenes with intron 1) are equivalent indicating that the origin (SV40 or BLG) of the 3'-poly A site does not affect levels of expression in this assay.

This previous analysis was performed on constructs which varied in components in addition to the HSA intron variations. In order to specifically analyze the effect of HSA intron number and position on levels of expression in this in vitro assay, constructs which vary only in HSA introns (Fig. 3A) were tested . All of these constructs contain the same BLG 5'-flanking sequences (promoter) with introduced SV40 enhancer as well as the same 3'-sequences (SV40 poly A site). A wide range of levels of expression are obtained from constructs with different HSA minigenes. As before, the very low level of HSA expression with the HSA cDNA (lane 1) is increased with the HSA minigene with intron 1 (lane 2) and further increased with introns 1 and 2 (lane 3). Inclusion of intron 2 alone (lane 9) has a similar effect as both introns 1 and 2 (lane 3) and clearly intron 2 is more efficacious than intron 1 alone (lane 2). This finding demonstrates that specific introns are more effective than others in providing expression of HSA. In addition, simply increasing the number of introns does not necessarily further increase expression as seen by the fact that the presence of the last 3 HSA introns, 12-14 (lane 6) results in a lower

level of expression than intron 2 alone (lane 9) but a similar level as with intron 1 alone (lane 2). This may be due to the nature of the specific introns and/or to their relative positions within the gene, with introns 12-14 at the 3'-end rather than the 5'-end. Significantly, similar and higher levels of expression are obtained with constructs containing either HSA minigenes containing the first 6 introns (introns 1-6, lane 4), the last 8 introns (introns 7-14, lane 7) or the entire HSA gene with all of its introns, 1-14 (lane 8). While the specific reasons for the increased and similar levels of expression obtained when using introns 1-6 or 7-14 are unknown, it is clear that the inclusion of either of these subsets results in expression as high as the inclusion of all HSA introns. The extremely high level of expression obtained with an HSA minigene containing introns 2 and 7-14 (lane 10) demonstrates the synergistic effects of specific intron combinations on levels of expression, and that expression of HSA can be increased several fold by incorporating these specific intron combinations as opposed to the inclusion of the entire gene with all of its introns.

The synergistic effects of specific HSA intron combinations on levels of expression of HSA (Fig. 3B) were investigated. The same relative levels of expression from constructs previously discussed, including the synergistic effect of introns 2 and 7-14 (lane 14) where expression is extremely high and much higher than what would result from an additive effect of intron 2 (lane 3) and introns 7-14 (lane 12). The combination of introns 1 and 7-14 (lane 13) was not synergistic since the level of expression supported by this construct is about the same as that supported by the construct with only introns 7-14 (lane 12). Additional synergistic combinations were also demonstrated. While the levels of expression from constructs with either HSA introns 1 (lane 2) or introns 12-14 (lane 8) are very low, introns 1 and 12-14 (lane 9) result in significantly higher levels than either alone or the additive effect of both together. The levels of expression due to the synergy between introns 2 and 12-14 (lane 10) were even higher. An even greater three part synergy involving introns 1 and 2 and 12-14 (lane 11) demonstrating levels of expression higher than that expected from an additive effect of the three alone or the additive effects of 1 with 12-14 and 2 or 2 with 12-14 and 1. The resultant level of expression with introns 1 and 2 and 12-14 was higher than with introns 1-6 (lane 6) or introns 7-14 (lane 12) or the entire gene with introns 1-14 (lane 5). The highest level of expression in these experiments was supported by a construct with HSA introns 2 and 7-14 (lane 14), similar to

introns 1 and 2 and 7-14 (lane 15). This was several fold higher than that supported by other constructs tested including one with the entire HSA gene with all 14 of its introns.

These results demonstrate that in living cells the level of expression of HSA is modulated by the specific complement of HSA introns, i.e., the number of HSA introns present in the construct, the specific introns incorporated, the relative location of introns, and the synergies between specific introns. Several fold higher levels of expression are obtained with constructs containing HSA minigenes with specific subsets of introns as compared with the entire HSA gene with all of its introns or with HSA cDNA.

Example 16

GENERATION AND IDENTIFICATION OF TRANSGENIC MICE

A. Collection of Fertilized Eggs

Mice used for the collection of fertilized eggs are the inbred line FBV/N, established at the NIH (Proc. Natl. Acad. Sci. USA 88:2065-2069, 1991). They were obtained from the National Institute of Health Animal Genetic Resource. To induce superovulation, 5-6 week females are injected with 5 i.u. of PMSG (Intervet), followed 44-48 hours later by injection of 5 i.u. of Human Chorionic Gonadotropin (HCG) (Sigma Chemical Company). The females are then mated with mature FBV/N males. The following morning, mated females are identified by the presence of vaginal plug. The flushing of fertilized eggs from the oviduct, treatment with hyaluronidase and culture conditions in M16 or M2 media are performed as described by Hogan, Costantini and Lacy "Manipulating and Mouse embryo, A laboratory manual" Cold Spring Harbor Laboratory (1986).

B. Preparation of DNA for Microinjection

To purify DNA sequences for microinjection, plasmids carrying the BLG or BLG/HSA genes were digested with Sal I. BLG and BLG/HSA sequences were separated from pGEM sequences by separation on 1.5% agarose gels,

electroelution and purification on elutip column (Schleicher & Schuell). DNA was suspended in 10 mM Tris pH 7.5 containing 0.5 mM EDTA at a concentration of 3 µg /ml and microinjected into the pronuclei of FBV/N eggs, which were subsequently implanted into the oviducts of CDI pseudopregnant recipient mice as described by Shani (Shani, 1985, Nature 314:283-286; Shani, 1986, Mol. Cell Biol. 6:2624-2631).

C. Microinjection

Injection pipettes are made from 10 cm long, 1.0 mm outside diameter, thin wall, borosillicate glass capillaries with filament (Cat. No. TW100F-4; World Precision Instruments, Inc. 375 Quinniplac Ave., New Haven Conn. 06513, USA). The holding pipettes are prepared from 9.0 cm long, 1.0 mm outside diameter glass capillaries (Cat. No. 105G; Drummond Scientific Co. 500 Pkwy., Broomall, PA 19008, USA), as described by Hogan, Costantini and Lacy "Manipulating the mouse embryo: A laboratory manual" CSHL (1986).

Microinjection is carried out in a drop of M42 medium overlaid with Silicone oil (Cat. No. 6428-R20; Thomas Scientific, P.O. Box 99, Swedesboro, NJ 08085-0099, USA), in a glass microscope slide chamber. The chamber is mounted on the microscope (Diaphot, Nikon) equipped with x20 and x40 differential interferences contrast (DIC) objectives and x10 eyepieces. 3D Hydraulic micromanipulators (Cat. No. MN-188, Nikon) are mounted on the stage of the microscope.

DNA (about 1ul) is introduced into the injection pipette at the broad side and it is carried to the tip by capillary action along the inner filament. The injection capillary is filled up with Flurinet FC77 (Cat. No. F4758; Sigma Chemical Company) and mounted onto the micromanipulator via the instrument collar (Cat. No. 070 321; Bunton Instrument Co. Inc. Rockville, MD 20850, USA), which is connected to the hydraulic drive unit (HDU; Bunton Instrument Co. Inc.; Rockville, MD 20850, USA) by a tubing (PE-100; Bunton Instrument Co. Inc.; Rockville, MD 20850, USA). The holding capillary is similarly mounted. The entire set up is filled with Flurinet FC77 (Sigma).

Batches of 20-30 pronuclear stage eggs are placed in the injection chamber. The holding and injection pipettes are brought to the chamber.

While the holding pipette picks up the egg, the injection pipette is inserted into the pronucleus and about 2 pl of the DNA solution is injected. When all the eggs in the chamber are injected, they are harvested and cultured for at least 1 hour before implantation.

D. Embryo Transfer

For routine embryo transfer outbred CD1 females mated with vasectomized CD1 males are used. Between 10-15 microinjected eggs are transferred to each oviduct, essentially as described by Hogan, Costantini and Lacy "Manipulating the mouse embryo: A laboratory manual" CSHL (1986).

E. Identification of Transgenic Mice

Transgenic animals were identified by tail biopsies (2 cm) taken 3 weeks after birth. Biopsies were incubated in 1 ml of 50 mM Tris pH 8.0 containing 0.5% SDS, 0.1 M EDTA and 200 µg proteinase K overnight at 55°C. Genomic DNA was purified from the homogenates by extraction with phenol/chloroform. Approximately 10 mg of DNA from each sample was digested with BamHI, fractionated on 0.8% agarose gel and transferred to Gene Screen filters (Du Pont). Hybridization was performed at 42°C in 50% formamide, with probe made from the insert of plasmid p598, 32P-CTP labeled using random primed DNA labeling kit (Boehringer Mannheim). Filters were washed with 0.2 x SSC containing 1% SDS at 60°C, and exposed to Kodak XAR-5 film at -80°C. (Fig. 4) Lanes are the analysis of DNA from transgenics #9 through #23 (followed by a blank (one) #25 and #26.

Example 17

ANALYSIS OF MAMMARY GLAND EXPRESSION

A. Collection and fractionation of milk.

Milk was collected from nursing transgenic mice 10-12 days after parturition. Three hours after mothers were separated from their pups they were injected intraperitonealy with 0.3 IU oxytocin (Sigma). Milk was collected 10 minutes later by gentle massage of the mammary gland and taken up in a

capillary tube (Clark et al.., 1987, Trends Biotechnology 5:20-24). Milk samples were diluted 1:5 in water containing 2 mM PMSF and Aprotinin (Sigma) and defatted by centrifugation. To prepare whey, the caseins were first precipitated by addition of 1 M HCl to pH 4.5. Whey proteins were subsequently precipitated in 10% trichloroacetic acid (TCA), washed with acetone and solubilized in SDS polyacrylamide gel electrophoresis (PAGE) sample buffer.

B. Milk protein analysis.

Milk proteins were analyzed for the presence of either sheep BLG or HSA. Diluted (1:5) and defatted milk collected from lactating G_0 or G_1 females from the seven transgenic lines (transgenic strain #30,35,37,38,39,40 and 41) generated from vector p585 were analyzed for the presence of sheep BLG by immuno-dot blot using rabbit anti-bovine BLG antibodies and iodinated protein A (Fig. 5A). The amount of material spotted on the nitrocellulose filters is indicated as well as the transgenic strain # from which the milk sample was obtained. C indicates control mouse milk. S indicates sheep milk sample. BLG indicates purified BLG protein. All seven transgenic lines expressed sheep BLG in their milk (Fig. 5A and Table 1). Expressed levels, ranging from about 1.0 mg/ml (lines #37 and #41) to about 8.5 mg/ml (lines #30, 35, and 39) were estimated from the intensity of the immuno-dot blot signals as compared with BLG standards and corrected for the dilution factor. As expected no signal was detected with control mouse milk which does not naturally contain BLG. In order to determine if levels of expression of BLG could be increased by increasing the length of 5'-sequences flanking the BLG transcription unit transgenic mice were produced from vectors p644 possessing approximately 5.5 kb of this region. Milk samples from two resultant transgenic lines, 46 and 48, were analyzed and found to express BLG at levels within the same range as obtained from transgenics produced from vector p585. Therefore, it appears that increasing the 5'-flanking region, containing regulatory sequences, from 3 kb (p585) to 5.5 kb (p644) or to 10.8 kb (p646) did not increase levels of expression of BLG.

For the detection of BLG, whey samples were fractionated on 15% SDS polyacrylamide gels. Proteins were either stained with Coomassie brilliant blue or transferred onto nitrocellulose filter in a Bio Rad trans-blot cell (Bio

Rad). Filters were blocked with TBS (20 mM TRIS/100 mM NaCl) containing 2% Bovine Serum Albumin (BSA, Sigma) and subsequently reacted for 2 hours with rabbit anti-BLG antiserum (Nordic Immunological Laboratories, Capistrano Beach, CA). The complex was incubated with goat anti-rabbit IgG (Bio Makor, Nes Ziona Israel) and then with rabbit peroxidase anti-peroxidase (PAP, Bio Makor). Peroxidase activity was revealed using diaminobenzidine as substrate. Alternatively, sheep BLG was detected using ^{125}I-protein A following the incubation with anti-BLG antiserum.

The whey fraction of milk obtained from the two highest expressing lines (30 and 35) were further analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot using anti-BLG antiserum. An immunoreactive band of approximately 18 kd was detected co-migrating with purified bovine BLG and native BLG in sheep milk (Fig. 5B) thus verifying the expression of authentic BLG in the milk of the transgenic lines. The amounts (mg or ml) of material loaded on the gel shown on Fig. 5B is indicated as well as the strain number from which the milk sample was obtained. C indicates control mouse milk. S indicates sheep milk sample. BLG indicates purified BLG protein. These results indicated that the basic transgenic vector with 3 kb of 5'-flanking sequences contained sufficient information to target high level expression of protein to the mammary gland of transgenic mice. A summary of results is shown in Table 2.

Table 2

EXPRESSION OF BLG

Vector	Strain	Expression*	Vector	Strain	Expression*
p585	30	8.4	p644	43	UD
	35	8.5		44	UD
	37	1.0		46	2.1
	38	6.0		48	4.2
	39	8.3		49	UD
	40	4.7			
	41	1.0	p646	52	1.0 - 2.0
				54	1.0 - 2.0
				56	UD

EXPRESSION OF HSA

Vector	Strain	Expression*	Vector	Strain	Expression*
p575	1-8	UD	p600	9,11,12,14,	UD
				16,17	UD
p598	15,18,21,25	UD	p599	19,20,22,24,	UD
	23	2.5		26	UD
p607	27,28	UD	p652	61	~6-10
	31	0.005		62	0.002
	34	0.001		63,65,67	UD
	36	0.035		66	0.002-0.04
	42	0.002		69	1.5
				71,72,73,74	UD**
				75	UD
p643	45,47	UD			
			p654	77	UD
p647	50,51,53	UD		76,78,79,80	UD**
	58,64	UD			
	59	0.002	p686	81	UD
				89	0.001
			p687	82	0.01
				83	5
				86	0.005
				84, 85 87,88	UD
p812	104	0.8-1.0	p696	90	UD
	103, 105	not yet		91	2.5
	106	determined		92	0.07
				93	0.002

* mg/ml ; **less than 0.001 mg/ml; "UD" means less than 0.001 mg/ml.
"Not yet determined" means that transgenic has been produced but the presence of HSA in the milk has not yet been determined.
"Low level" means that preliminary evaluation by dot blot suggested the presence of HSA in the milk of transgenic animals. Subsequent quantitation of the level of HSA in the milk relied on a level of 0.001 mg/ml or greater to be considered detectable.

HSA was detected in milk samples fractionated on 7.5% SDS or native polyacrylamide gels. Proteins were either stained with Coomassie blue or transferred onto nitrocellulose filters. The filters were blocked with 3% gelatin at 37°C and then reacted overnight at room temperature with iodinated anti-HSA monoclonal antibodies. After extensive washings with TBS containing 0.5% tween, filters were exposed to Kodak XAR-5 film at -80°C. The initial attempt to produce transgenic mice expressing HSA in their milk was by introducing the HSA cDNA into the 5'-untranslated region of the first exon of the BLG gene of vector p585, resulting in vector p575 (Fig. 2A). The milks of lactating females from 8 transgenic lines produced from vector p575 were analyzed for the presence of HSA by immuno-dot blot using iodinated anti-HSA monoclonal antibodies. None of the 8 lines secreted detectable levels of the human protein (Tables 1 and 2). It appeared that although the BLG vector was able to drive expression of its own BLG gene, it was unable to support the expression of the inserted HSA cDNA. Therefore, a series of vectors was tested in which the sheep BLG promoter was fused to HSA minigenes possessing either their first or first and second introns within their native sites of the HSA cDNA (Fig. 2A). Vector p599 differs from vector p575 only by the presence of HSA intron 1. Vector p600 also includes an HSA minigene with intron 1, but has had the BLG coding sequences deleted though it maintains the untranslated BLG exon 7 with its polyadenylation signal and site as well as BLG 3'-flanking sequences. In vector p598, with an HSA minigene containing intron 1, BLG coding sequences, exon 7 and 3'-flanking sequences were deleted and replaced with an SV40 polyadenylation signal and site. Vector p607 is similar to p600 except that it includes both HSA introns 1 and 2.

From a total of 16 individual transgenic lines produced from vectors with an HSA minigene with intron 1 (p599, p600, p598), only one (#23 from p598) expressed detectable levels of HSA in its milk (Fig. 6A and Table 2). The top row represents the spotting of the indicated amounts (ng) of commercially purified HSA (Sigma). The middle and bottom rows represent the spotting of milk samples from the indicated transgenic mouse strains, control mouse (C), human milk (H) and sheep milk(S). The milk from line 23 was estimated to contain about 2,000-3,000 µg /ml HSA as determined by comparison of its signal with HSA standards in the immuno-dot blot (Fig. 6B). The top row represents the spotting of the indicated amounts(ng) of purified HSA. The middle and bottom rows represent the spotting of indicated amounts of milk

samples from indicated transgenic mouse strains, control mouse milk (C) and human milk (HM). Significantly, four of the six transgenic lines produced from vector p607, containing an HSA minigene with its first 2 introns, expressed detectable levels of HSA in their milk, ranging from 1 to 35 µg /ml (Table 1). Several transgenic strains generated from vector p652, which contains an HSA minigene with introns 1-6, expressed HSA in their milk. One of these strains expressed 1.5 mg/ml while a second expressed 6-7 mg/ml.

Similar levels of HSA expression were supported by constructs which contained the same HSA minigene whether they possessed the SV40 or BLG poly A site.

Milk samples from the 5 expressing lines, as identified by immuno-dot assay were subjected to SDS-PAGE and immunoblot (Fig. 6C). In the figure, HSA represents analysis of commercial HSA. HM represents human milk. HSA 23 represents analysis of milk sample from transgenic strain #23. An immunoreactive band co-migrating with purified HSA (65 kd) was detected in the milk of all immuno-dot positive lines. Densitometry of the autoradiograms confirmed the quantitative estimates of HSA based upon the immuno-dot blot. Mouse milk contains a significant amount of endogenous mouse serum albumin which co-migrates with human serum albumin in SDS-PAGE gels. However, as demonstrated in the immuno-detection assays (Fig. 7A and 7B), the anti-HSA monoclonal antibody specifically detected the human protein and not the mouse protein. In Fig. 7A and 7B, HM represents human milk, HSA represents commercially purified HSA (Sigma) and C represents control mouse milk. The human and mouse proteins were also distinguishable by their distinct electrophoretic mobilities on native polyacrylamide gels. Milk from expressing line 23 clearly contains both human (low mobility) and mouse (high mobility) albumin as seen by generalized protein staining with Coomassie (Fig. 7A). The lower mobility band was confirmed to be HSA by native gel and immunoblot analysis (Fig. 7B). A summary of the expression is shown in Table 2.

C. Expression of HSA RNA in different tissues of transgenic mice.

In order to examine the tissue specificity of expression of HSA RNA total RNA was isolated from various tissues of transgenic female mice on day 10-12

of lactation. Total RNA from various tissues of transgenic lactating mice was isolated by the LiCl/Urea procedure. RNA (10-15 mg) was fractionated on MOPS/formaldehyde agarose gels and blotted onto a nylon filter and hybridized to a ^{32}P-labeled anti-sense RNA probe synthesized with the RNA labeling kit (Boehringer Mannheim), according to the supplier's protocol, using HSA cDNA in pSK (Stratagene) plasmid. The HSA probe crosshybridizes to endogenous mouse serum albumin mRNA in liver.

Two patterns of HSA RNA expression were observed as represented by line 23, produced from vector p598, whose milk contains large quantities of HSA and line 19, produced from vector p599, whose milk contains no detectable HSA (Fig. 8). B represents brain; H represents heart; K represents kidney; L represents liver; Lu represents lung; M represents mammary gland; S represents spleen; and SK represents skeletal muscle. In line 23 transcripts of the transgene were clearly detected in the mammary gland and to a lesser extent in skeletal muscle. No detectable signal was found in the other tissues examined, even after a long exposure of the autoradiogram. The HSA transgene RNA migrated slightly slower than the endogenous mouse serum albumin mRNA (2070 ribonucleotides). This is consistent with an expected transgenic mRNA size of about 2230 ribonucleotides composed of the untranslated portion of BLG exon 1 from its cap site to the site of introduction of the HSA minigene, the HSA transcription unit itself minus introns 1 sequences removed by splicing, and SV40 sequences upstream of its polyadenylation site.

In mouse line 19, as well as six of the transgenic lines carrying vector p575 (all of whose milk contains no HSA), transgene transcripts were not detected in the mammary gland. However, significant levels of transcripts were found in the kidney. Their higher mobility than the endogenous mouse albumin mRNA indicates an RNA smaller than the size expected (2783 ribonucleotides) of a polycistronic mRNA composed of both HSA and BLG sequences as would be produced from vectors p599 and p575. Endogenous mouse serum albumin mRNA was also detected in the kidney of control mice.

D. <u>In situ</u> hybridization

In situ hybridization was performed on paraffin sections of mammary glands of virgin and lactating transgenics and control mice as described in Sassoon, D., Lyons, G., Wright, W., Lin, V., Lassar, A., Weintraub, H. and Buckingham, M. 1989, Nature 341:303-307. The probe used was ^{35}S-UTP labeled antisense RNA synthesized from the HSA cDNA with T7 polymerase. (Fig. 9) Panel A shows a section of lactating mammary gland of transgenic strain HSA #23. Alveoli are marked "AL". Panel B shows the same section as A viewed under dark-field illumination. Panel C shows a section of virgin mammary gland of transgenic mouse strain #23; panel D is a dark field view of the same section. A duct is marked "Du". Panel E shows a section of mammary gland from a control non-transgenic mouse; and panel F shows the same section under dark-field illumination.

E. Explant studies

Explant cultures of mammary glands of virgin and lactating mice were performed as described by Pittius, CW., Sankaran, L., Topper, YJ., and Hennighausen, L. 1988. Mol. Endocrinol 2:1027-1032. Briefly, after mincing, pieces of approximately 1 mm were cultivated on lens paper floats in serum free M199 medium. For hormonal stimulation, bovine insulin (0.1 or 5 µg /ml), hydrocortisone (0.1 or 5 µg /ml) and ovine prolactin (1 or 5 µg /ml) were added to the medium. All hormones were purchased form Sigma (ST. Louis, MO). The medium was collected for several days, and then screened for the presence of HSA or other milk proteins. (Fig. 10) HSA represents commercially purified HSA signal. C represents control mouse explants. I represents insulin; P represents prolactin; F represents hydrocortisone. First set IP, IFP, IF, I) represent treatments with the lower concentrations of hormones. Second set represent treatments with the higher concentration of hormones.

Example 18

IMMUNOHISTOCHEMICAL DETECTION OF HSA AND β-CASEIN IN MAMMARY GLANDS OF TRANSGENIC AND CONTROL MICE

For immunohistochemical staining, mammary glands were fixed in 4% paraformaldehyde at 4°C for 16 hrs. The fixed material was dehydrated in a

series of 70-100% ethanols, cleared in chloroform and embedded into paraffin. Sections (5 μM) were cut on a paraffin microtome and mounted on 3-aminopropyltrietoxysilane-treated slides. Mounted sections were deparaffinized in xylene (2 changes, 5 min each), processed through a 100-70% series of ethanols, washed in PBS (5 min) and incubated for 10 min in absolute methanol containing 0.3% H_2O_2 for the inhibition of endogenous peroxidase activity. The sections were then immersed in PBS for 5 min and incubated for 30 min in PBS containing 3% normal goat serum. The sections were incubated sequentially with the following reagents: (I) rabbit anti-HSA antiserum diluted 1:1000 or rabbit anti-mouse β-casein antiserum diluted 1:1000, or normal rabbit serum diluted 1:1000; (2) goat anti-rabbit IgG antiserum diluted 1:100; (3) rabbit peroxidase-antiperoxidase (PAP) complex diluted 1:500. Each incubation (45 min) and all the dilutions were prepared in PBS containing 3% normal goat serum and 3% normal mouse serum. Between treatments the slides were washed with 3 changes (5 min each) of PBS. After the final washing the peroxidase activity was revealed by incubating the sections in saline buffered with 0.05 M Tris-HCl (pH 7.4) containing 0.05 M imidazole, 0.05% diaminobenzidine-HCl and 0.01% H_2O_2 for 1-3 min. The sections were briefly washed in distilled water, lightly counter stained with hematoxylin, dehydrated through ascending ethanols, cleared in xylene and coverslipped.

Figure 11 shows the pattern of HSA (A, B) and casein (C, D) immunostaining of sections of virgin (A, C) and lactating (B, D) transgenic mice of strain #23 (which express HSA in their milk). In virgin transgenic glands casein staining is concentrated in the luminal surfaces of epithelial cells lining the ducts, a pattern similar to that found with control non-transgenics (Figure 13), while HSA staining is concentrated not only in the apical part of the epithelial cells but is also found over the epithelial cell cytoplasm. In lactating glands HSA and casein co-localize to the apical parts of the epithelial cells.

Figure 12 shows the pattern of HSA (A, B) and casein (C, D) immunostaining of sections of virgin (A, C) and lactating (B, D) transgenic mice of strain #69 (which also express HSA in their milk). In virgin glands both HSA and casein staining are confined to the luminal surfaces of epithelial cells, while in lactating glands there is a striking difference between the two patterns.

In some ducts only a small proportion of the cells are positive for HSA whereas casein staining is uniform and all the epithelial cells are stained.

Figure 13 shows the pattern of HSA (A, B) and casein (C, D) immunostaining in sections of virgin (A, C) and lactating (B, D) control non-transgenic mice. As expected there is no detectable staining with the anti-HSA antiserum. The pattern of staining of casein is similar to that found with the transgenic strains.

Example 19

CROSSBREEDING OF TRANSGENIC STRAINS WHICH EXPRESS HSA IN MILK RESULTS IN DOUBLE TRANSGENICS WHICH EXPRESS HIGHER LEVELS OF HSA THAN EITHER PARENT STRAIN

A crossbreeding was performed between transgenic strain #23 (generated from vector p598, expresses 2.5 mg/ml of HSA in milk) and strain #69 (generated from vector p652, expresses 1.5 mg/ml of HSA in milk) resulting in a double transgenic strain, #1000, which possesses both transgenes. Quantitation by dot blot immunoanalysis, as previously described, demonstrated that strain #1000 females produce milk with HSA at a concentration of 4-5 mg/ml.

Example 20

CULTURE AND ANALYSIS OF MAMMARY EXPLANTS

Explants were prepared from the abdominal and thoracic mammary glands essentially as described by Topper *et al.*. (Meth. Enzymol 39, 443-454, 1975), with the following modifications. Explants were maintained in medium 199, pH 7.2, containing 25 mM HEPES, 2.2 g/liter $NaHCO_3$, 200,000 IU/liter penicillin, 200 mg/liter streptomycin and 10 mg/liter neomycin. Sixteen to twenty explants, 1-2 mm^3 in size, were incubated for the indicated time period on impregnated lens paper, floated in 35 mm culture dishes with 2 ml of medium containing the indicated concentrations of insulin, hydrocortisone and prolactin in various combinations. Media was changed daily.

Proteins were analyzed either by Western immunoblot analysis or metabolic labeling. For Western immunoblot analysis aliquots from the collected medium were precipitated with 10% TCA, and washed with ethanol and acetone. Pellets were dissolved in SDS-PAGE sample buffer and were fractionated on 7.5% SDS polyacrylamide gels. Proteins were either stained with Coomassie blue or transferred onto nitrocellulose filters in a Bio-Rad trans-blot cell (Bio Rad). Filters were blocked with TBS (20 mM Tris, pH 7.5, 500 mM NaCl) containing 3% gelatin for 3 h at 37°C and subsequently reacted overnight with iodinated anti-HSA monoclonal antibodies at room temperature. After extensive washings with TBS containing 0.5% tween, the filters were exposed to Kodak XAR-5 film at -80°C. For BLG detection, membranes were reacted with anti-BLG antibody, then with anti-rabbit IgG, and extrAvidin-alkaline Phosphatase conjugate. The complex was stained with NBT/BCIP (Bio-Makor Nes Ziona Israel) according to supplier instructions. De novo synthesized proteins were metabolically labeled as follows. Thirty minutes before labeling, medium was changed to methionine-free DME medium. The explants were then incubated for 4.5 hours in 1 ml methionine-free medium containing 150 µCi/ml ^{35}[S]-methionine. The collected explants were extracted with 50 mM Tris-HCl, pH 8.0, 5 mM NaCl, 0.5% Nonidet P-40 (NP-40), 3 µg/ml PMSF and 1% aprotinin. Following extraction, lysates were clarified for 10 min at 10,000 x g and total protein synthesis was determined as TCA insoluble radioactivity. Fractions (300-500 µl) of explant lysates or medium containing 0.5 x 10^6 or 0.25 x 10^6 cpm, respectively, were pre-absorbed on Protein A-Sepharose CL-4B beads (Pharmacia Fine Chemicals, Uppsala, Sweeden) for 30 min. Immunoprecipitation was performed with rabbit anti-human serum albumin polyclonal antibody or rabbit anti-BLG antiserum (Nordic Immunology, Tilburg, The Netherlands) for 1-2 hour at 0°C, followed by binding to Protein A-Sepharose CL-4B beads for 30 min at 0°C. After extensive washing with 50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 5 mM EDTA, 0.5% NP-40 and 5% sucrose, the pellets were washed once with the same buffer without NP-40 and sucrose, suspended in SDS-PAGE sample buffer, electrophoresed on 7.5 % polyacrylamide gel and exposed to Kodak XAR-5 films, following flourography.

Example 21

EXPRESSION OF HSA, BLG AND β-CASEIN RNA AND PROTEIN IN THE MAMMARY GLANDS OF VIRGIN, PREGNANT AND LACTATING MICE

The expression of HSA, BLG and β-casein RNAs in mammary glands was determined through development from virgin (V) (8-12 weeks old), pregnant (P) at various days of pregnancy and lactating (L) animals. Total RNA was extracted from mammary glands of transgenic mice carrying an HSA transgene (strain #23), a native sheep full length BLG transgene (strain #35) and from control non-transgenic mice. RNA (10 μg) were analyzed by Northern blot analysis using the corresponding radiolabeled probes (Figure 14) Virgin mice of strain #23 expressed significant amounts of HSA mRNA. A somewhat higher level was found on the 5th day of pregnancy. However, there was a significant increase in its amount on day 12 that continued to increase up to day 18 of pregnancy. This latter level was higher than that found during lactation. In contrast, in strain #35 BLG transgene RNA began to accumulate only on day 12 of pregnancy and its level continued to increase during pregnancy and lactation. A pattern similar to that of the BLG transgene was seen for the expression of RNA from the endogenous β-casein gene in transgenics and in control mice (although a decrease in β-casein RNA level was observed in lactating mice of strain #23). The synthesis and secretion of HSA from mammary explants of strain #23 and #69 and of BLG from explants of strain #35 generally follow the pattern of RNA expression (Figure 15). De novo synthesized proteins were metabolically labeled for 4.5 hours immediately after explantation, immunoprecipitated with anti-HSA antibodies and analyzed as previously described. However, in strain #23 the level of HSA synthesized in virgin explants was comparable to that seen in 18 day pregnant mouse explants with a dramatic decrease in HSA synthesis and secretion observed between virgin explants and 5 day pregnancy explants. Explants from lactating mice synthesize less HSA compared to those from 18 day pregnant transgenics mimicking the pattern seen in RNA expression. No BLG synthesis was detected in explants of virgin transgenics. BLG accumulated on day 12 of pregnancy as did BLG RNA.

Example 22

HORMONAL CONTROL OF HSA AND BLG EXPRESSION AND SECRETION FROM MAMMARY EXPLANTS OF VIRGIN TRANSGENICS

In order to determine the effects of different hormone combinations on the synthesis and secretion of HSA and BLG from mammary explants of virgin mice, cultured over time, de novo synthesized proteins were metabolically labeled immediately upon explant (d0) or on the 5th day of culture (d5). Following metabolic labeling, approximately half of the explants from each sample were collected and 2 ml of fresh medium 199 containing about 1000-fold excess of cold methionine chase was added to the remaining explants. Collected explants and media were processed and immunoprecipitated as previously described. The remaining explants were processed similarly after 24 hours of chase (d1 and d6, respectively). Aliquots of media and lysates representing equal tissue weight were analyzed by SDS-PAGE and fluorography. The results are shown in Figure 16 with the top part showing the analysis of explants from transgenic strain #23 and the bottom, transgenic strain #35. Figure designations are: -, no hormones; I, insulin; P, prolactin; F, hydrocortisone.

HSA was produced and secreted from mammary explants on day 0 in the presence or absence of hormones, while BLG was not produced under either condition immediately upon explant. In the presence of insulin and prolactin, explants cultured for 5 days continued to produce and secrete HSA. Hydrocortisone had a minor effect. The combination of insulin and prolactin, with or without hydrocortisone, appears to have had a stabilizing effect on expressed HSA since a higher proportion was observed after 24 hours of cold chase on day 6 than on day 1. BLG production and secretion was observed only on day 5 of culture and only in the presence of insulin and prolactin. Hydrocortisone had a minor effect. We have also found that the production of β- and α1-casein in BLG transgenics and control mice followed the hormonal control pattern of BLG in BLG transgenic strain #35 (data not shown). These milk proteins were also induced by a combination of insulin and prolactin. However, we found the production of caseins from explants of HSA transgenic strain #23 on day 0.

Example 23

CORRELATION BETWEEN LEVELS OF EXPRESSION OF HSA IN MILK AND LEVELS OF EXPRESSION AND SECRETION OF HSA FROM MAMMARY

EXPLANTS OF VIRGIN FEMALES AMONG DIFFERENT STRAINS OF TRANSGENIC MICE

In order to determine whether we could use levels of expression of HSA from mammary explants of virgin transgenic females to predict whether specific strains of transgenics will ultimately express HSA in their milk, and to estimate that level, upon lactation, we investigated several explant culture parameters. In a series of experiments we determined the best time points, culture conditions and means of estimation of HSA production from explants, that will best reflect the in vivo secretion of HSA in the milk of several transgenic strains. Figure 17 shows a Western immunoanalysis of HSA secreted into milk of a number of BLG/HSA hybrid gene transgenic mouse strains. Three ul of defatted diluted (1:5) milk samples were analyzed on SDS PAGE. Proteins were blotted onto nitrocellulose membrane and reacted with iodinated anti-HSA monoclonal antibodies as previously described. Relative levels of HSA in each milk were estimated in relative densitometry scan units. Figure 18 shows the results of an explant experiment where mammary explants from various HSA transgenic strains were metabolically labeled either immediately upon explantation, or on day 5 in culture in M-199 medium containing insulin (5 ug/ml) and prolactin (5 ug/ml). Explant lysates and media were pooled from duplicate plates which contained explants from 3-4 virgin animals. Equal amounts of either total TCA precipitable protein cpm from the medium or lysate (A), or amounts of medium or lysate representing equal tissue weight (B) were analyzed by immunoprecipitation with anti-HSA antibodies, SDS-PAGE, fluorography and densitometric scanning of autoradiographs as previously described. Levels of HSA were represented in relative densitometry scan units.

The correlations (r values) between HSA secretion in the milk and levels of expression from explants are summarized in Table 3. From this table it can be seen that there is a relatively high correlation between HSA secretion to the milk and HSA synthesis and secretion in explants at days 0 or 5 of culture. Mammary explants from mouse strains which do not secrete HSA in their milk also do not produce and secrete HSA from their explants. Explants from mice which secrete high levels of HSA in their milk do produce the HSA in culture. While it appears that any of the conditions tested produce high correlations, it appears that the best parameters for this work is to culture explants from

transgenics for 5 days in the presence of insulin and prolactin (5 µg/ml each) and to measure and compare ^{35}S-labeled HSA levels secreted to the medium on the basis of equal amount of TCA precipitable protein. Figure 19 shows the graph comparing levels of expression under these conditions.

These data suggest that this type of explant expressional analysis can be used on a biopsy of a virgin transgenic farm animal mammary gland in order to forecast the potential of that animal to express the transgene protein, as well as to estimate the level of expression, in its milk later in its development, upon lactation.

TABLE 3

Correlation (r values) between HSA secretion in milk and synthesis and secretion by mammary explants of virgin animals[1]

Parameter[1]	Parameter[2]	Day[3]	Basis for Analysis	Correlation[4] (r value)
HSA in milk	HSA in media	0	Equal cpm/lane	0.79
HSA in milk	HSA in lysates	0	Equal cpm/lane	0.83
HSA in milk	HSA in media	5	Equal cpm/lane	0.93
HSA in milk	HSA in lysates	5	Equal cpm/lane	0.87
HSA in milk	HSA in media	0	Equal tissue weight/lane	0.87
HSA in milk	HSA in lysates	0	Equal tissue weight/lane	0.90
HSA in milk	HSA in media	5	Equal tissue weight/lane	0.92
HSA in milk	HSA in lysates	5	Equal tissue weight/lane	0.89

[1]Transgenic strains #76, 81, 86, 92, 69, 83, 23 and 1000 were evaluated.
[2]Expression of HSA expressed by explants as determined in either media or lysates.
[3]Day in culture of explant upon analysis.
[4]Correlations (r values) were determined using Sigmaplot software.

Example 24

PRODUCTION OF TRANSGENIC GOATS

A. Induction of Superovulation

Embryos were recovered from Saanen goat does that have been induced to superovulate by treating them for 12 days with intravaginal sponges impregnated with 30 mg of fluorogestone acetate (Chrono-Gest, Intervet International B.V.-Boxmeer-Holland). From the evening of the 9th day after sponge insertion, 5 intramuscular injections of follicle stimulating hormone (FSH-P, Schering Corp.) were administered every 12 hours (5, 4, 3, 3 and 2 mg FSH-P). On the evening of the 11th day when the last FSH injection was administered, the sponges were withdrawn. Does were checked for estrous every 4 to 12 hours, beginning 12 hours after sponge removal. They were mated by 2 bucks at 20 and 36 hours after sponge withdrawal. One to two cell eggs were collected about 62 hours after sponge removal. The recipients are similarly synchronized with intravaginal sponges. On the evening of the 11th day sponges are removed and the recipient does are injected with 500 units of PMSG (Intervet).

B. Surgery

Does were taken off food (36 hours) and water (12 hours) prior to surgery. Anesthesia is induced by intravenous injection of thiopentone sodium and maintained by mixtures of oxygen (1-2 liters/minute) and halothane (1-2%) (Halocarbon Laboratories, N. Augusta, SC.). The reproductive tract is exposed through mid-central incision and a glass catheter inserted into the oviduct through the fimbria. Five ml PBS containing 5% FCS were introduced into the uterine lumen through a blunted 18-gauge needle and forced through the uterotubal junction and along the oviduct.

C. Microinjection

DNA (1-4 μg /ml) is injected into one pronucleus of 1 or 2 cell eggs placed in a chamber filled with ovum culture medium (Flow Labs, Irving, Scotland) containing 20% FCS and covered with Flurinet 70 (Sigma).

Pronuclei are visualized using the Nikon Diaphet Inverted microscope equipped with Nomarski optics at x400. Eggs are microinjected essentially as described by Hogan et al.. (Manipulating the mouse embryo- a laboratory manual, Cold Spring Harbor Laboratory, 1986). Surviving embryos were surgically transferred to the oviduct of recipient does using the Socorex 1-5 µl micropipette. Up to 10 embryos are transferred to each recipient.

Example 25

HSA EXPRESSION FROM EXPLANTS OF MAMMARY GLANDS OF AN ABORTED TRANSGENIC BABY GOAT AND A DEAD NEWBORN TRANSGENIC BABY GOAT

Two female transgenic goats were produced. However, one (goat #1) was spontaneously, prematurely, aborted about 1-2 weeks before expected delivery and found approximately 24 hours after it had aborted. Upon finding, it was stored at 4°C. Laboratory tests indicated that the mother suffered from Q-fever (a disease caused by rickettsia that damages the placenta, but not the embryo, and leads to spontaneous abortion). Goat #2 was also spontaneously, prematurely, aborted about 1 week before expected delivery, lived for approximately 24 hours and then died for unknown reasons. It was also stored at 4°C. Its mother did not appear to be ill. A few hours after goat #1 was found and the death of goat #2, a sample of their mammary tissue was removed into a small container filled with M-199 medium. Samples were cut into smaller pieces (explants) which were incubated on impregnated lens paper in 35 mm tissue culture flasks with 2 ml of medium in the absence or presence of 5 µg/ml, each, of insulin (I), hydrocortisone (F) and prolactin (P). Medium was replaced and collected every 24 hours and stored at -70°C. Aliquots (300 µl) were lyophilized, solubilized in sample buffer and analyzed on SDS-PAGE gels. The proteins were either blotted onto nitrocellulose membrane for Western immunoassay (Figure 20A) or stained with Coomassie brilliant blue (Figure 20B). The nitrocellulose membrane was blocked with 3% gelatin in TBS buffer (10 mM Tris, 500 mM NaCl) and hybridized overnight with iodinated anti-HSA monoclonal antibodies in TBS buffer containing 1% gelatin and 0.2% Tween 20. The membrane was washed in the same buffer and exposed to XAR film at -70°C.

Figure 20 shows that an immunoreactive protein of the same size of purified HSA was secreted from explants of goat #1 during the first 24 hours of culture in the presence of IFP. By day 3 of incubation HSA was reduced to just at or below the level of detection. We had also found that mammary explants of BLG/HSA transgenic mice also expressed high levels of HSA during the first 24 hours of culture but that this declined greatly with time in culture. We could not detect secretion of caseins from explants of goat #1, although β-lactoglobulin appears to be expressed in the medium of explants from the first day of culture in the presence of IFP (Figure 20B).

Mammary explants from goat #2 did not secrete detectable levels of HSA during the first day in culture or during subsequent days (Figure 20B). Traces of two other milk proteins could be detected, but may represent a non-specific hybridization due to the overloading of proteins. While β-lactoglobulin was detected in the media of explants of goat #1 from the first day of culture, β-lactoglobulin was not clearly detected until the 6th day of culture from explants of goat #2.

Deposit of Strains Useful in Practicing the Invention

Deposits of biologically pure cultures of the following strains were made under the Budapest Treaty with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, The accession numbers indicated were assigned after successful viability testing, and the requisite fees were paid.

Access to said cultures will be available during pendency of the patent application to one determined by the Commissioner of the United States Patent and Trademark Office to be entitled thereto under 37 C.F.R. §1.14 and 35 U.S.C. §122, or if and when such access is required by the Budapest Treaty. All restriction on availability of said cultures to the public will be irrevocably removed upon the granting of a patent based upon the application and said cultures will remain permanently available for a term of at least five years after the most recent request for the furnishing of samples and in any case for a period of at least 30 years after the date of the deposits. Should the cultures become nonviable or be inadvertently destroyed, they will be replaced with viable cultures(s) of the same taxonomic description.

Strain/Plasmid	ATCC No.	Deposit Date
p652.2	68653	July 25, 1991
p696.9	68654	July 25, 1991

One skilled in the art will readily appreciate the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The peptides, antibodies, methods, procedures and techniques described herein are presented as representative of the preferred embodiments, or intended to be exemplary and not intended as limitations on the scope of the present invention. Changes therein and other uses will occur to those of skill in the art which are encompassed within the spirit of the invention or defined by the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Hurwitz, David R
Nathan, Margret
Shani, Moshe

(ii) TITLE OF INVENTION: Transgenic Protein Production

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Rhone-Poulenc Rorer, Inc.
(B) STREET: 500 Virginia Ave., Bldg. 3A
(C) CITY: Ft. Washington
(D) STATE: Pennsylvania
(E) COUNTRY: USA
(F) ZIP: 19034

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Goodman, Rosanne
(B) REGISTRATION NUMBER: 52,534
(C) REFERENCE/DOCKET NUMBER: A0856-US

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (215) 962-4130
(B) TELEFAX: (215) 962-4107

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19557 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Minghetti, P P
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Kuang, W.-J.
Dennison, O E
Hawkins, J W
Beattie, W G
Dugaiczyk, A

(B) TITLE: MOLECULAR STRUCTURE OF THE HUMAN ALBUMIN GENE IS REVEALED BY NUCLEOTIDE SEQUENCE WITHIN Q11-22 OF CHROMOSOME 4
(C) JOURNAL: J. Biol. Chem.
(D) VOLUME: 261
(F) PAGES: 6747-6757
(G) DATE: 1986
(K) RELEVANT RESIDUES IN SEQ ID NO:1: FROM 1 TO 19002

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GCTGGGATTA CAGGCATGAG CCACTGTGCC CAGCCGACAG ATACTATTAT TATTTCCATT 12780

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TTTTACCTTA GAAAAAACAA TTCTAAAATT CATATGGAAT CAAAAAAGAG CCTGCAG 19557

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 50, line 16 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet [X] [3]

Name of depositary institution [4]

American Type Culture Collection

Address of depositary institution (including postal code and country) [5]

12301 Parklawn Drive
Rockville, Maryland 10852 U.S.A.

Date of deposit [6]	Accession Number [6]
July 25, 1991	68653

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

Escherichia coli, p652.2

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. [ ] This sheet was received with the international application when filed (to be checked by the receiving Office)

Helen Bell
(Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 55 line 11 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet [X] [3]

Name of depositary institution [4]

American Type Culture Collection

Address of depositary institution (including postal code and country) [5]

12301 Parklawn Drive
Rockville, Maryland 10852 U.S.A.

Date of deposit [6]	Accession Number [7]
July 25, 1992	68654

B. ADDITIONAL INDICATIONS [1] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

Escherichia coli, p696.9

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [2] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [4] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. [ ] This sheet was received with the international application when filed (to be checked by the receiving Office)

Helen Bell
(Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

WHAT IS CLAIMED AS NEW AND IS DESIRED TO BE COVERED UNDER LETTERS PATENT IS:

1. A DNA construct comprising a 5' flanking sequence from a mammalian gene and a sequence coding for human serum albumin, wherein the human serum albumin sequence comprises at least one, but not all, of the introns in the naturally occurring gene encoding for the HSA protein.

2. The construct of claim 1 wherein said gene is a milk protein gene.

3. The construct of claim 1 wherein said introns are selected to provide for expression of HSA in mammalian cells at levels equal to or greater than the naturally occurring HSA gene.

4. The genetic construct of claim 3 wherein said introns are selected from the group consisting of 1-6, 7-14, 1+7-14, 1 + 2 +12-14, 2 + 7-14 and 1+ 2 + 7-14.

5. A DNA construct encoding HSA, comprising one but not all of the first 7 introns of the HSA gene, and one of the last 7 introns of the HSA gene.

6. A DNA construct encoding HSA comprising two contiguous exons encoding HSA and an HSA intron.

7. A DNA construct comprising DNA sequences encoding human serum albumin operably linked to a mammary tissue specific promoter, said DNA construct expressed by the mammary glands of a lactating female transgenic mammal.

8. A transgenic mammal having incorporated into its genome the DNA construct of claim 7.

9. The transgenic mammal of claim 8 wherein the mammal is selected from the group consisting of mice, rabbits, sheep, goats, pigs and cattle.

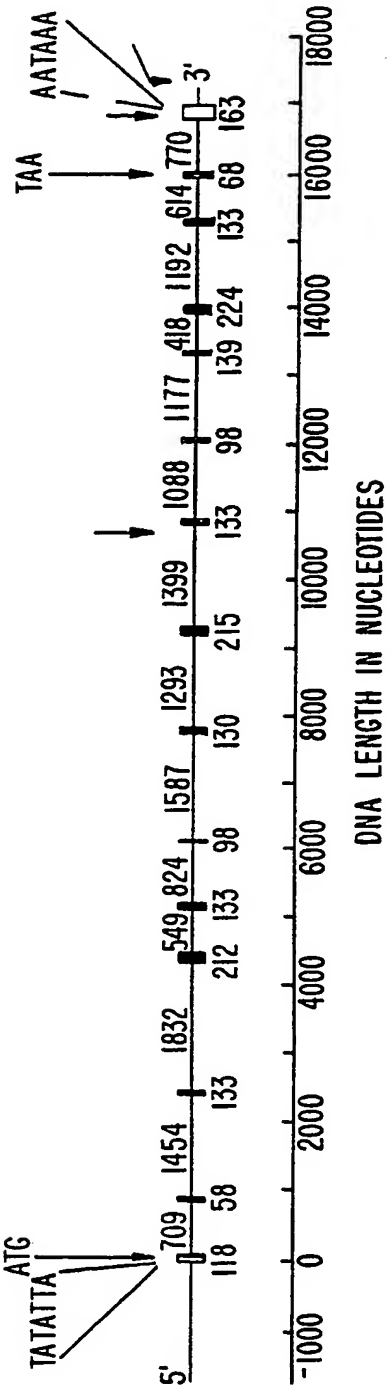
10. The transgenic mammal of claim 8 wherein the promoter is the ß-lactoglobulin protein promoter.

11. The transgenic mammal of claim 8 wherein said mammal is a mouse having incorporated into its genome DNA construct encoding human serum albumin that is produced in the milk of a lactating mouse at levels of approximately 100 µg/ml of milk or greater.

12. A method of making a transgenic mammal having incorporated into its genome a DNA construct encoding human serum albumin and a mammary tissue specific promoter, said DNA construct expressed by mammary glands of a lactating female transgenic mammal comprising providing a DNA construct containing the ß-lactoglobulin promoter operably linked with a nucleotide sequence encoding human serum albumin.

13. The method of claim 12 further comprising microinjecting the DNA construct into the embryo of a mammal selected from the group consisting of mice, rabbits, sheep, goats, pigs and cattle.

14. The method of claim 13 further comprising testing the animals for production of human serum albumin in the milk of lactating females and mating the animals containing the highest level of human serum albumin in the milk.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1399
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1177
139
418
224
1192
133
614
68
770
163
-1000
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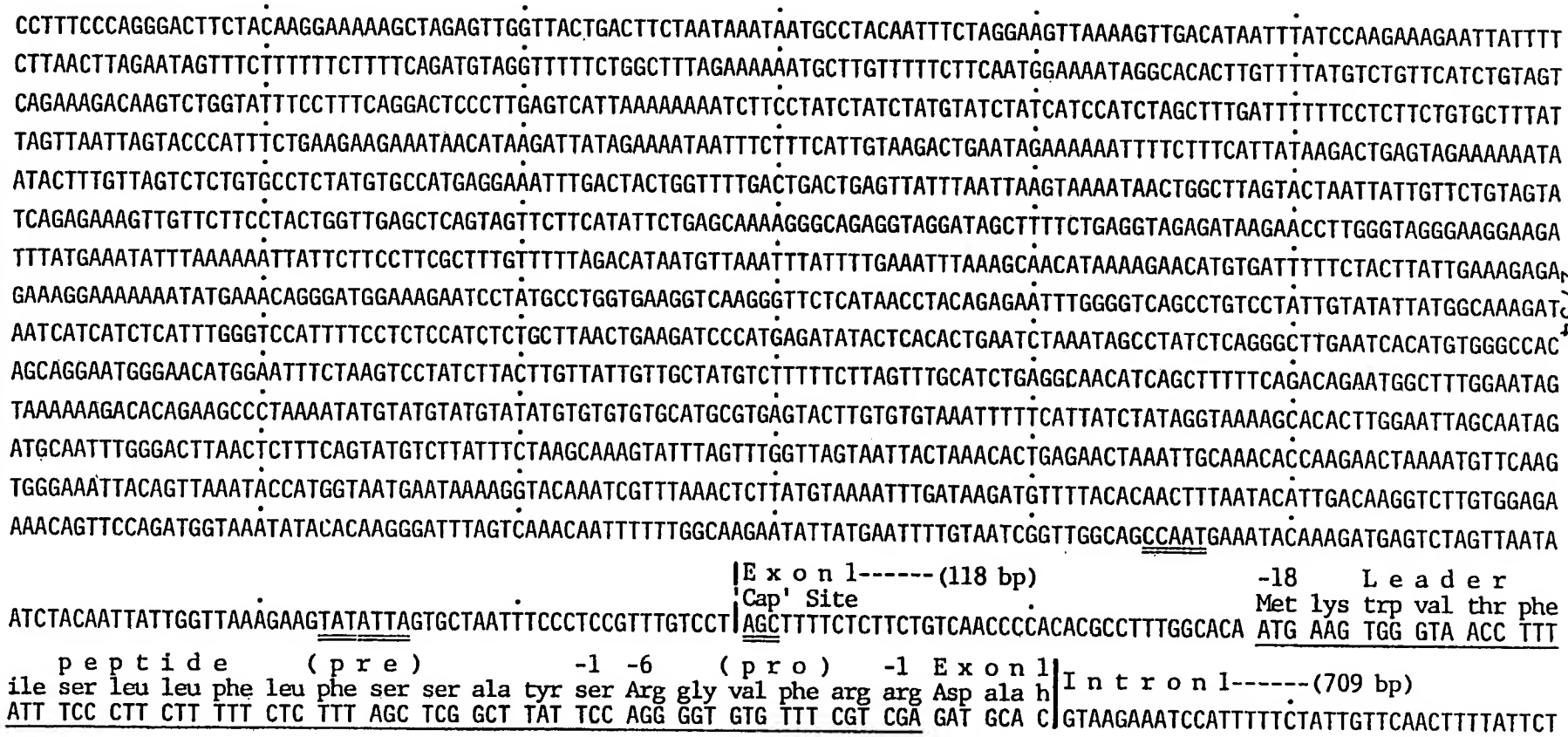
14000
16000
18000
DNA LENGTH IN NUCLEOTIDES

FIG. 1A

FIG. IB-1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Exon1------(118 bp)
'Cap' Site
-18 Leader
Met lys trp val thr phe
ATCTACAATTATTGGTTAAAGAAGTATATTAGTGCTAATTTCCCTCCGTTTGTCCTAGCTTTTCTCTTCTGTCAACCCCACACGCCTTTGGCACA ATG AAG TGG GTA ACC TTT
peptide (pre) -1 -6 (pro) -1 Exon1
Intron1------(709 bp)
ile ser leu leu phe leu phe ser ser ala tyr ser Arg gly val phe arg arg Asp ala h
ATT TCC CTT CTT TTT CTC TTT AGC TCG GCT TAT TCC AGG GGT GTG TTT CGT CGA GAT GCA C
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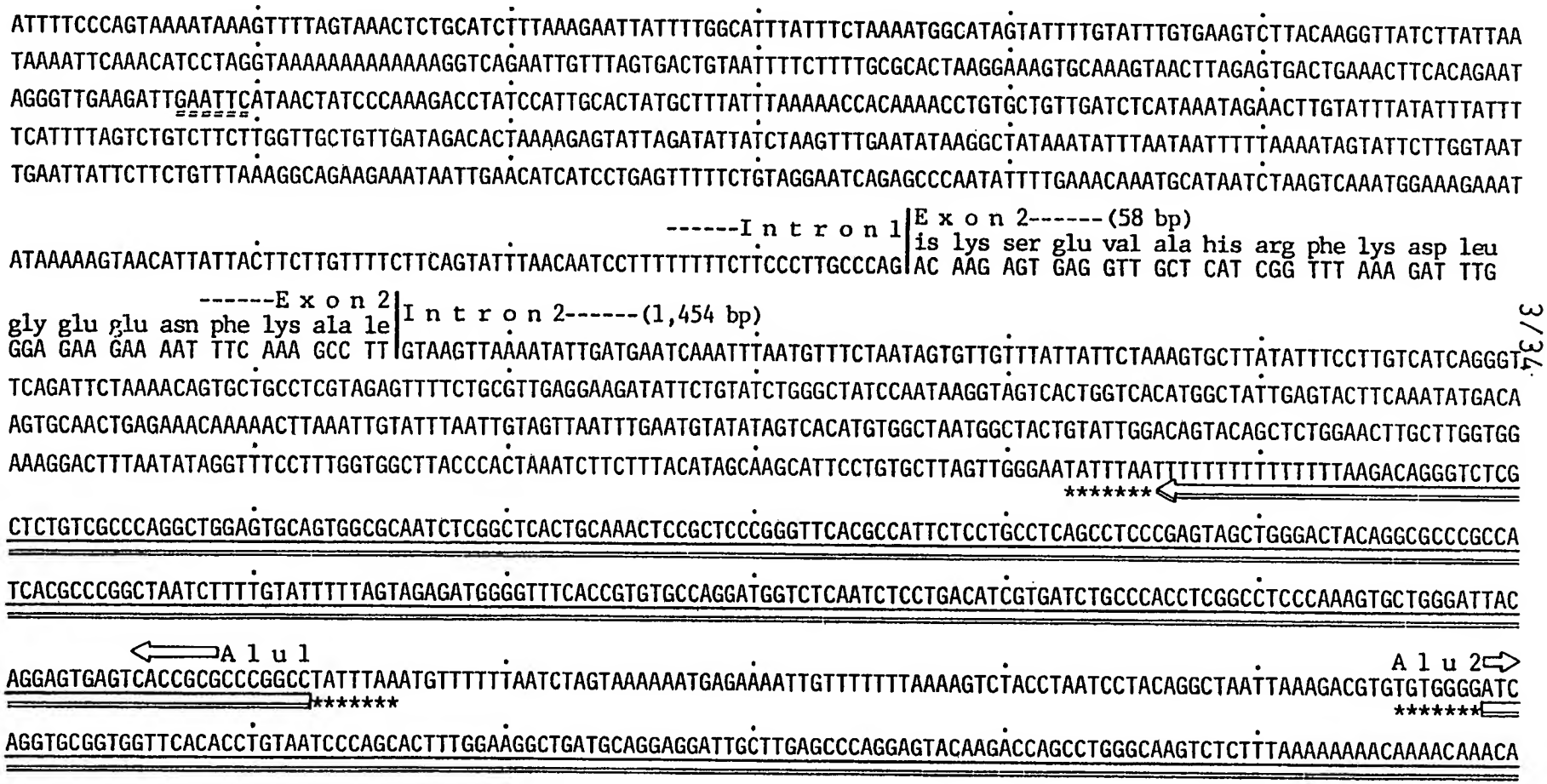
FIG. IB-2



SUBSTITUTE SHEET

FIG. IB-3

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                                                                                                  *******

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TCTAGGCCTCAGATCATACCTGATATGAATAGGCTTTCTGGATAATGGTGAAGAAGATGTATAAAAGATAGAACCTATACCCATACATGATTTGTTCTCTAGCGTAGCAACCTGTTACAT

                                  ------I n t r o n 2|E x o n 3------(133 bp)
                                                      u val leu ile ala phe ala gln tyr leu gln gln cys pro phe glu asp his val
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                                                                                                  ------E x o n 3|I n t r o n 3---
lys leu val asn glu val thr glu phe ala lys thr cys val ala asp glu ser ala glu asn cys asp lys ser leu
AAA TTA GTG AAT GAA GTA ACT GAA TTT GCA AAA ACA TGT GTT GCT GAT GAG TCA GCT GAA AAT TGT GAC AAA TCA CTT|GTAAGTACATTCTAAT
---(1,832 bp)
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FIG. IB-4

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TGAATGTTTTTCTCCCCTAGGTGAATTCACCTGCTGCTTAGAAGCTTATTTTCTCTTGATTTCTGTTATAATGATTGCTCTTACCCTTTAGTTTTAAGTTTCAAAATAGGAGTCATATAA
CTTTCCTTAAAGCTATTGACTGTCTTTTTGTCCTGTTTTATTCACCATGAGTTATAGTGTGACAGTTAATTCTTATGAAAATTATATAGAGATGGTTAAATCATCAGAAACTGTAAACCT
CGATTGGGAGGGGAAGCGGATTTTTAAATGATTTCCTGACCAAGCTTAACCAGTATATTAAATCCTTTGTACTGTTCTTTGGCTATAAAGAAAAAAGGTACTGTCCAGCAACTGAAACCT
```

```
---Intron 3|Exon 4------(212 bp)
           |his thr leu phe gly asp lys leu cys thr val ala thr leu arg glu thr tyr gly glu met ala asp cys cys ala
GCTTTCTTCCATTTAG|CAT ACC CTT TTT GGA GAC AAA TTA TGC ACA GTT GCA ACT CTT CGT GAA ACC TAT GGT GAA ATG GCT GAC TGC TGT GCA

lys gln glu pro glu arg asn glu cys phe leu gln his lys asp asp asn pro asn leu pro arg leu val arg pro glu val asp val
AAA CAA GAA CCT GAG AGA AAT GAA TGC TTC TTG CAA CAC AAA GAT GAC AAC CCA AAC CTC CCC CGA TTG GTG AGA CCA GAG GTT GAT GTG

                                    ------Exon 4|Intron 4------(549 bp)
met cys thr ala phe his asp asn glu glu thr phe leu lys ly|
ATG TGC ACT GCT TTT CAT GAC AAT GAA GAG ACA TTT TTG AAA AA|GTAAGTAATCAGATGTTTATAGTTCAAAATTAAAAAGCATGGAGTAACTCCATAGGCCAAC
ACTCTATAAAAATTACCATAACAAAAATATTTTCAACATTAAGACTTGGAAGTTTTGTTATGATGATTTTTTAAAGAAGTAGTATTTGATACCACAAAATTCTACACAGCAAAAAATATG
ATCAAAGATATTTTGAAGTTTATTGAAACAGGATACAATCTTTCTGAAAAATTTAAGATAGACAAATTATTTAATGTATTACGAAGATATGTATATATGGTTGTTATAATTGATTTCGTT
TTAGTCAGCAACATTATATTGCCAAAATTTAACCATTTATGCACACACACACACACACACACACACTTAACCCTTTTTTCCACATACTTAAAGAATGACAGAGACAAGACCATCATGTGC
AAATTGAGCTTAATTGGTTAATTAGATATCTTTGGAATTTGGAGGTTCTGGGGAGAATGTCGATTACAATTATTTCTGTAATATTGTCTGCTATAGAAAAGTGACTGTTTTTCTTTTTCA
```

FIG. IB-5


Intron 4|Exon 5------(133 bp)
s tyr leu tyr glu ile ala arg arg his pro tyr phe tyr ala pro glu leu leu phe phe ala lys arg tyr lys ala ala
AAATTTAG|A TAC TTA TAT GAA ATT GCC AGA AGA CAT CCT TAC TTT TAT GCC CCG GAA CTC CTT TTC TTT GCT AAA AGG TAT AAA GCT GCT
------Exon 5|Intron 5------(824 bp)
phe thr glu cys cys gln ala ala asp lys ala ala cys leu leu pro lys
TTT ACA GAA TGT TGC CAA GCT GCT GAT AAA GCT GCC TGC CTG TTG CCA AAG|GTATTATGCAAAAGAATAGAAAAAAAGAGTTCATTATCCAACCTGATTTTGT
CCATTTTGTGGCTAGATTTAGGGAACCTGAGTGTCTGATACAAACTTTCCGACATGGTCAAAAAAGCCTTCCTTTTATCTGTCTTGAAAATCTTTCATCTTTGAAGGCCTACACTCTCGT
TTCTTCTTTTAAGATTTGCCAATGATGATCTGTCAGAGGTAATCACTGTGCATGTGTTTAAAGATTTCACCACTTTTTATGGTGGTGATCACTATAGTGAAATACTGAAACTTGTTTGTC
AAATTGCACAGCAAGGGGACACAGTTCTTGTTTATCTTTTCATGATAATTTTTAGTAGGGAGGGAATTCAAAGTAGAGAATTTTACTGCATCTAGATGCCTGAGTTCATGCATTCATTCC
ATAAATATATATTATGGAATGCTTTATTTTCTTTTCTGAGGAGTTTACTGATGTTGGTGGAGGAGAGACTGAAATGAATTATACACAAAATTTAAAAATTAGCAAAATTGCAGCCCCTGG
GATATTAGCGTACTCTTTCTCTGACTTTTCTCCCACTTTTAAGGCTCTTTTTCCTGGCAATGTTTCCAGTTGGTTTCTAACTACATAGGGAATTCCGCTGTGACCAGAATGATCGAATGA
TCTTTCCTTTTCTTAGAGAGCAAAATCATTATTCGCTAAAGGGAGTACTTGGGAATTTAGGCATAAATTATGCCTTCAAAATTTAATTTGGCACAGTCTCATCTGAGCTTATGGAGGGGT
------Intron 5|Exon 6------(98 bp)
leu asp glu leu arg asp glu gly lys ala ser ser ala lys gln arg leu
GTTTCATGTAGAATTTTTCTTCTAATTTTCATCAAATTATTCCTTTTTGTAG|CTC GAT GAA CTT CGG GAT GAA GGG AAG GCT TCG TCT GCC AAA CAG AGA CTC
------Exon 6|Intron 6------(1,587 bp)
lys cys ala ser leu gln lys phe gly glu arg ala phe lys ala tr
AAG TGT GCC AGT CTC CAA AAA TTT GGA GAA AGA GCT TTC AAA GCA TG|GTAAATACTTTTAAACATAGTTGGCATCTTTATAACGATGTAAATGATAATGCTTCA
GTGACAAATTGTACATTTTTATGTATTTTGCAAAGTGCTGTCAAATACATTTCTTTGGTTGTCTAACAGGTAGAACTCTAATAGAGGTAAAAATCAGAATATCAATGACAATTTGACATT
ATTTTTAATCTTTTCTTTTCTAAATAGTTGAATAATTTAGAGGACGCTGTCCTTTTTGTCCTAAAAAAAGGGACAGATATTTAAGTTCTATTTATTTATAAAATCTTGGACTCTTATTCT
AATGGTTCATTATTTTTATAGAGCTGTAGGCATGGTTCTTTATTTAATTTTTTAAAGTTATTTTTAATTTTTGTGGATACAGAGTAGGTATACATATTTACGGGGTATATGAGATATTTT
GATATAAGTATACAACATATATAATCCCTTTATTTAATTTTATCTTCCCCCCAATGATCTAAAACTATTTGCTTGTCCTTTTATGTCTTATAGTTAAATTCAGTCACCAACTAAGTTGAA
GTTACTTCTTATTTTTGCATAGCTCCAGCTCTGATCTTCATCTCATGTTTTTGCCTGAGCCTCTGTTTTCATATTACTTAGTTGGTTCTGGGAGCATACTTTAATAGCCGAGTCAAGAAA

FIG. IB-6

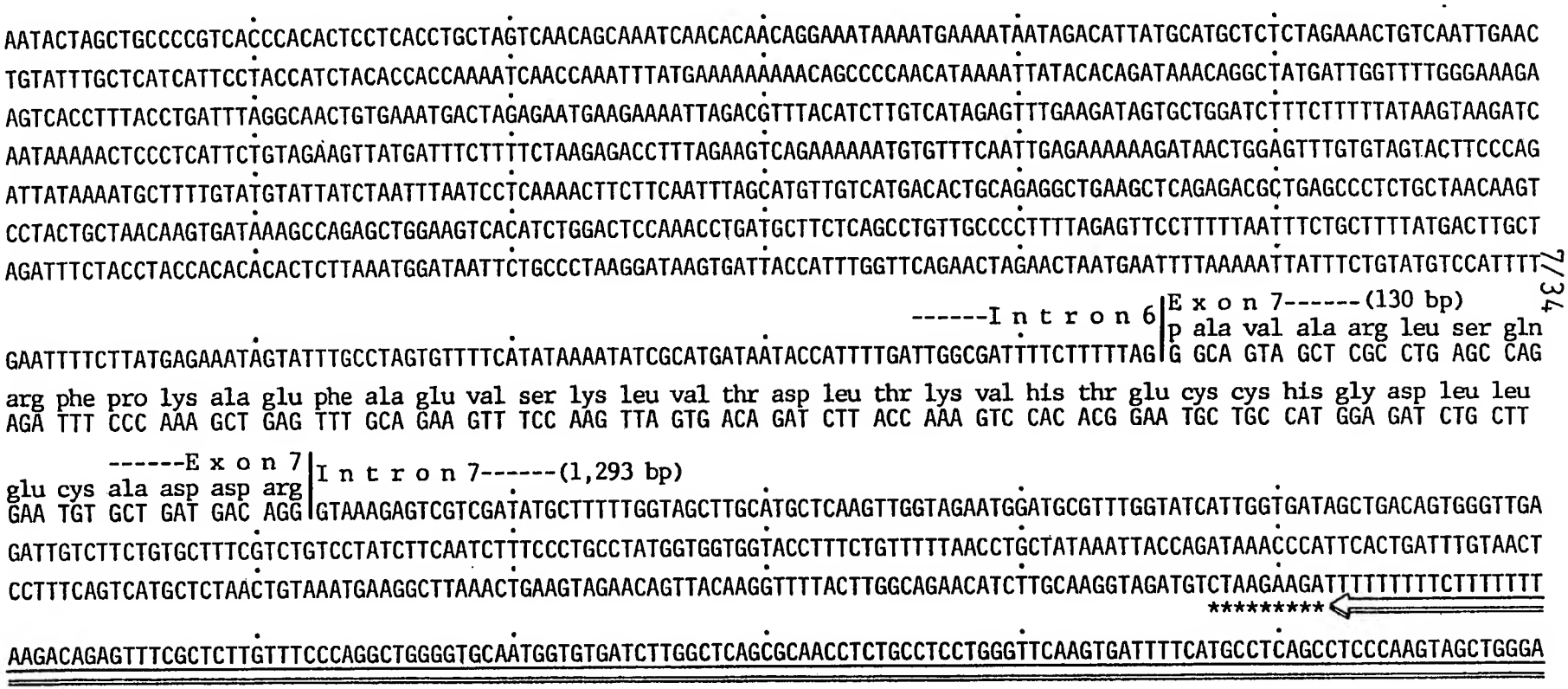
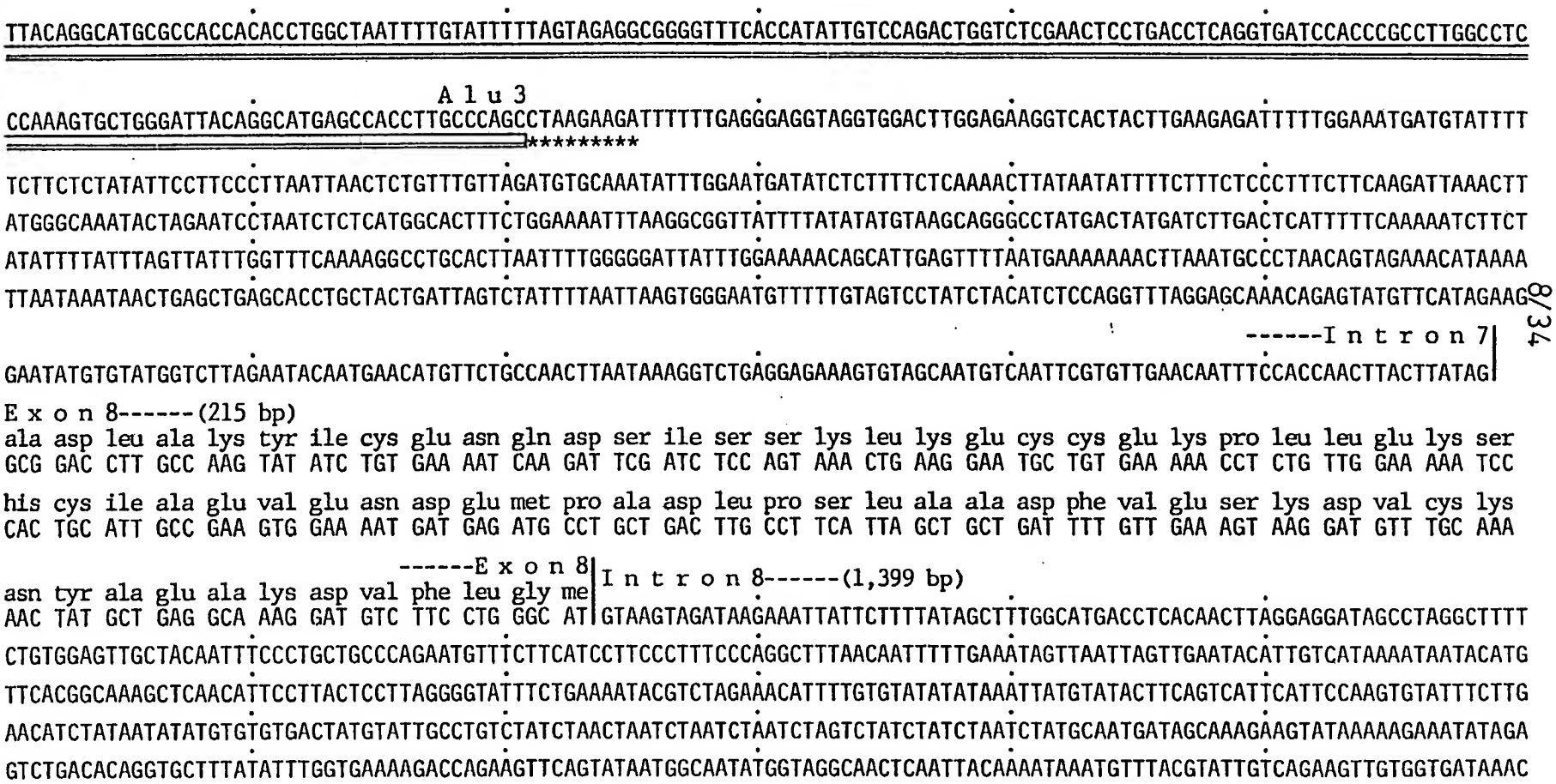
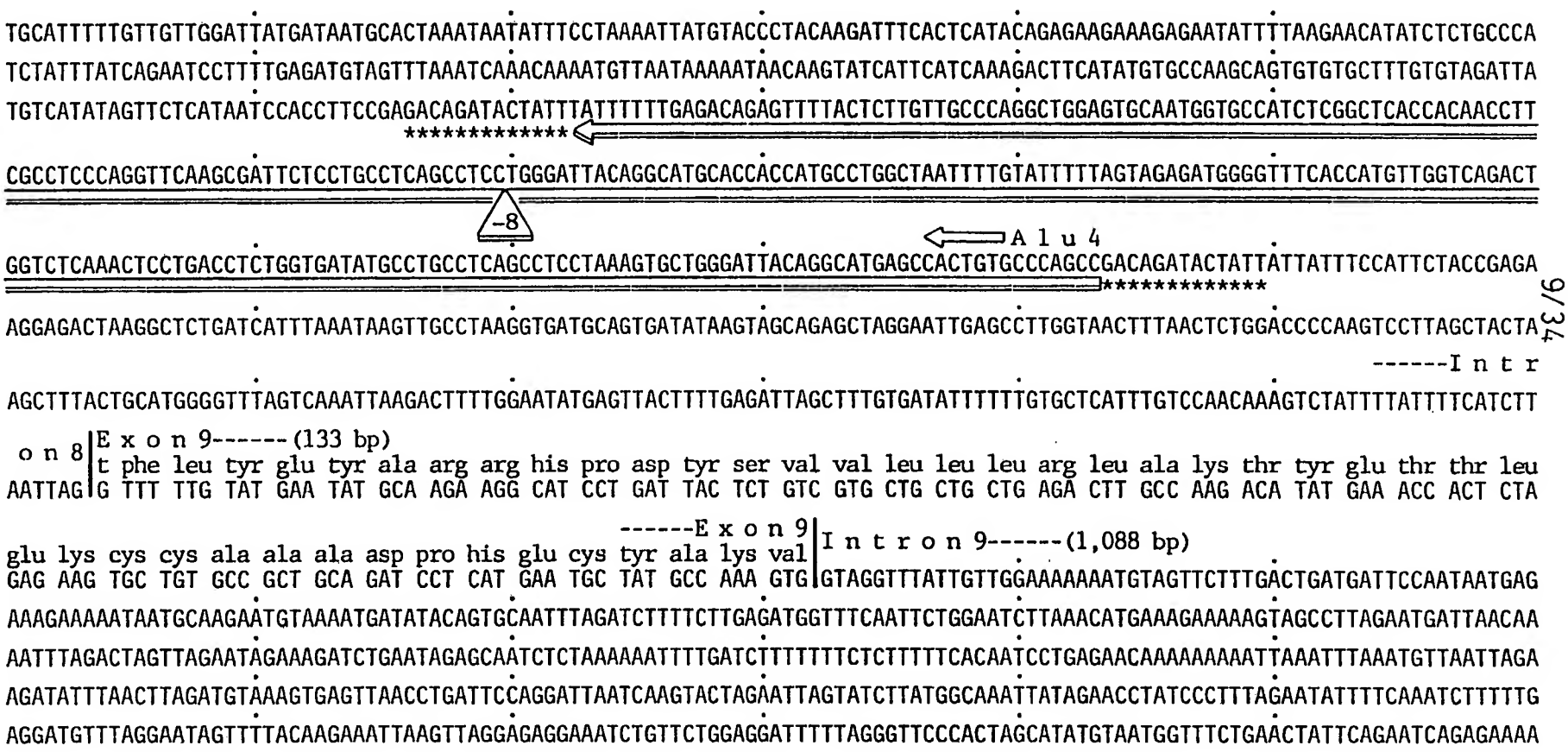


FIG. IB-7



SUBSTITUTE SHEET

FIG. IB-8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TCTATTTATCAGAATCCTTTTGAGATGTAGTTTAAATCAAACAAAATGTTAATAAAAATAACAAGTATCATTCATCAAAGACTTCATATGTGCCAAGCAGTGTGTGCTTTGTGTAGATTA
TGTCATATAGTTCTCATAATCCACCTTCCGAGACAGATACTATTTATTTTTTGAGACAGAGTTTTACTCTTGTTGCCCAGGCTGGAGTGCAATGGTGCCATCTCGGCTCACCACAACCTT
CGCCTCCCAGGTTCAAGCGATTCTCCTGCCTCAGCCTCCTGGGATTACAGGCATGCACCACCATGCCTGGCTAATTTTGTATTTTTAGTAGAGATGGGGTTTCACCATGTTGGTCAGACT
-8
Alu 4
GGTCTCAAACTCCTGACCTCTGGTGATATGCCTGCCTCAGCCTCCTAAAGTGCTGGGATTACAGGCATGAGCCACTGTGCCCAGCCGACAGATACTATTATTATTTCCATTCTACCGAGA
AGGAGACTAAGGCTCTGATCATTTAAATAAGTTGCCTAAGGTGATGCAGTGATATAAGTAGCAGAGCTAGGAATTGAGCCTTGGTAACTTTAACTCTGGACCCCAAGTCCTTAGCTACTA
------Intr
AGCTTTACTGCATGGGGTTTAGTCAAATTAAGACTTTTGGAATATGAGTTACTTTTGAGATTAGCTTTGTGATATTTTTTGTGCTCATTTGTCCAACAAAGTCTATTTTATTTTCATCTT
on 8 Exon 9------(133 bp)
AATTAG t phe leu tyr glu tyr ala arg arg his pro asp tyr ser val val leu leu leu arg leu ala lys thr tyr glu thr thr leu
G TTT TTG TAT GAA TAT GCA AGA AGG CAT CCT GAT TAC TCT GTC GTG CTG CTG CTG AGA CTT GCC AAG ACA TAT GAA ACC ACT CTA
------Exon 9
glu lys cys cys ala ala ala asp pro his glu cys tyr ala lys val
GAG AAG TGC TGT GCC GCT GCA GAT CCT CAT GAA TGC TAT GCC AAA GTG
Intron 9------(1,088 bp)
GTAGGTTTATTGTTGGAAAAAAATGTAGTTCTTTGACTGATGATTCCAATAATGAG
AAAGAAAAATAATGCAAGAATGTAAAATGATATACAGTGCAATTTAGATCTTTTCTTGAGATGGTTTCAATTCTGGAATCTTAAACATGAAAGAAAAAGTAGCCTTAGAATGATTAACAA
AATTTAGACTAGTTAGAATAGAAAGATCTGAATAGAGCAATCTCTAAAAAATTTTGATCTTTTTTTCTCTTTTTCACAATCCTGAGAACAAAAAAAAATTAAATTTAAATGTTAATTAGA
AGATATTTAACTTAGATGTAAAGTGAGTTAACCTGATTCCAGGATTAATCAAGTACTAGAATTAGTATCTTATGGCAAATTATAGAACCTATCCCTTTAGAATATTTTCAAATCTTTTTG
AGGATGTTTAGGAATAGTTTTACAAGAAATTAAGTTAGGAGAGGAAATCTGTTCTGGAGGATTTTTAGGGTTCCCACTAGCATATGTAATGGTTTCTGAACTATTCAGAATCAGAGAAAA

FIG. IB-9

```
CTCATTTTTCCTGCTTTCAAGAAGCTACTGTATGCCAGGCACCATGCACAAACAATGACCAACGTAAAATCTCTCATTTTGGAGAGCCTGGAATCTAACTGGAAAGGTGAACTAATAATA
ATAATATGTACAATCATAGCCATCATTTATTAAACTTTTATTATATGCAAGGCACTGTTTAATTTCATTAGCTTACCTGGTTTACAGAGCAGCTCTATGAGATGAGTGCCATCTTTGCCC
CTATTTTAGGGATAAGGATTCCGAAATGTGGAGATGGTAAGTAAAATTGCACAACTGAAGAATGAGTTACATGACTTGGCTCAAATACTGGTCATTGAACTCCAGAGCCTGAATATTCTT
AACCACTTACATGATGCAAGCTCACCAAATAAATAGTTCGAATGTATTGTGACAGAGCGGCATTGATATTCATCTATTCATGTGGCTTTGAGTAGGAAGAAGAAAGGATATCATTCTGAC
                                                    ------Intron 9|Exon 10------(98 bp)
                                                                  |phe asp glu phe lys pro leu val glu glu pro gln
CAGAGGGGTGAAAAACAACCTGCATCTGATCCTGAGGCATAATACTATTAACACAATTCTTTTATGTTTCAG|TTC GAT GAA TTT AAA CCT CTT GTG GAA GAG CCT CAG
                                                                  ------Exon 10|Intron 10------(1,177 bp)
asn leu ile lys gln asn cys glu leu phe glu gln leu gly glu tyr lys phe gln asn al|
AAT TTA ATC AAA CAA AAT TGT GAG CTT TTT GAG CAG CTT GGA GAG TAC AAA TTC CAG AAT GC|GTAAGTAATTTTTATTGACTGATTTTTTTTATCAATT
TGTAATTATTTAAGACTTAATATATGAGCCACCTAGCATAGAACTTTTAAGAATGAAAATACATTGCATATTTCTAATCACTCTTTGTCAAGAAAGATAGGAGAGGAGAGATAAAATAGT
TGATGGGGTGGAGAGGTCTATATTTGAATGTAGTCTAAAAATTGTTCTCTTAAGATTGGAAGTATGTAGGCTGGGAGGGTAAATACCAAATCTTGGTATATCAGAACTGAGCATGTCCCT
TGAAGGTTAAGAAATAGTTAATGGGCAAATAGAGCATGGCAATATTTTGTAGAGCAGCAAGTAGTAGGCCTTGAATAGATGTCGCTCAAAAAGTAATATGTAAGCTGAACACAAAAATGT
AACAAATGAATTTAGATACATATTTGAATATTAAATTCAGGTTGTTTGGGAGATGCACCTAGTCTTTGATGGTTAAACCTTTCCCTCCATAGAAGAGACAGAGACAGAATGGCTTGCTGG
ACTAATGTCCCAATTCAATAGAGTCTTATCTACGAAGGTTAAAAACAAGAAGAGACATATTATACAGTAGATATTTATTGTGTGGCTCATACACATGGTGCTCTTCTGATTATGGATTTT
AGAGATAATAACAGTGAACAAGACATAGTTTCTTTCCTCGAGTAGATTAAAGTCATACATTGACTTTTAATGGTGACTGGCATTCTTAATACATGATTATTATATATTAGGTACCATGTC
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GAAGCTCTCTCCAGGTTTTATTGAAAGAAGAAATTAATAAATTTATTAATGTCACTGAATTAGGCAACTCACTTTCCCAAGATTATGCAAGTGGTACAGGTGGAACTCAAAGCCAAGTTT
AACTAGTTGTTCAGGAGAATGTTTTCTACCCTCCACTAACCCACTACTCTGCAGATGGAGATAATATGATGAATGGAACATAGCAACATCTTAGTTGATTCCGGCCAAGTGTTCTCTGTT
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FIG. IB-10

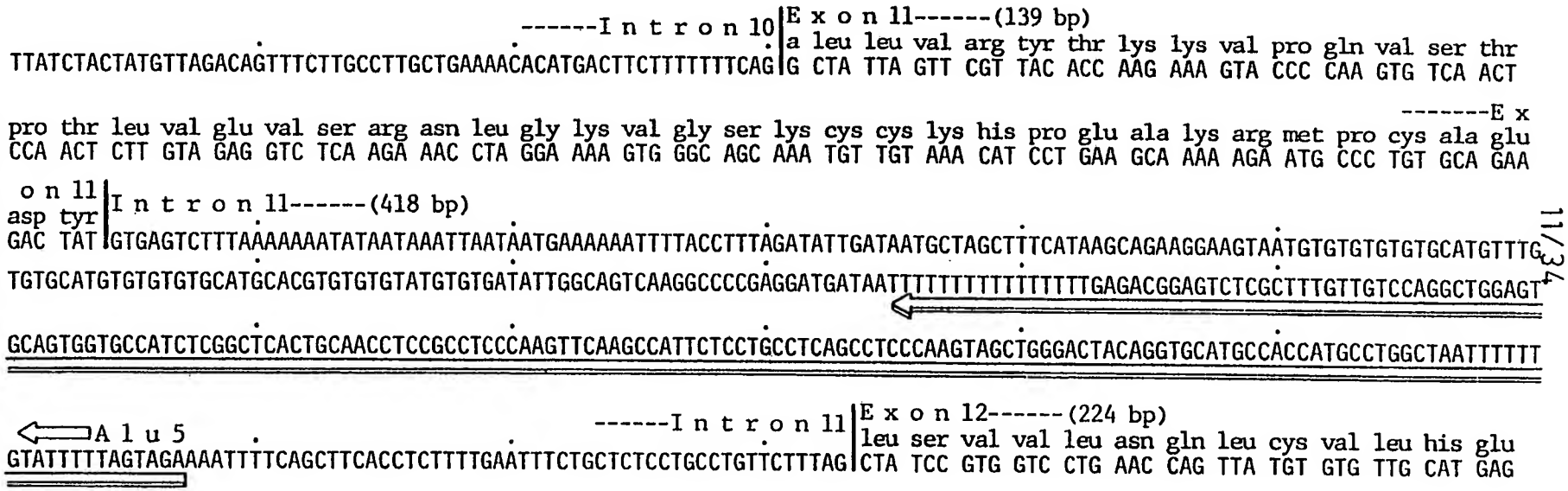

FIG. IB-II

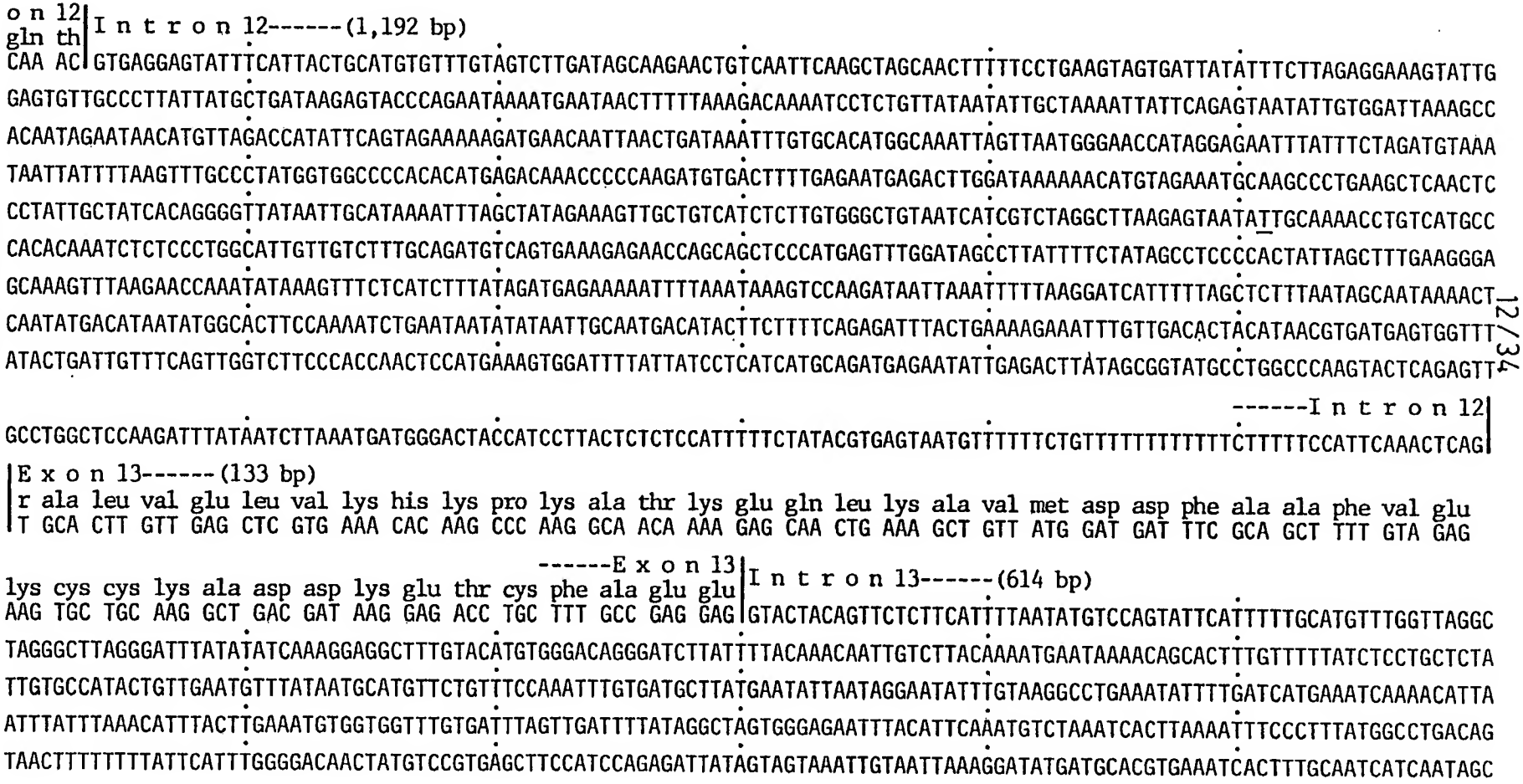
on 12
gln th
Intron 12------(1,192 bp)
CAA AC
GTGAGGAGTATTTCATTACTGCATGTGTTTGTAGTCTTGATAGCAAGAACTGTCAATTCAAGCTAGCAACTTTTTCCTGAAGTAGTGATTATATTTCTTAGAGGAAAGTATTG
GAGTGTTGCCCTTATTATGCTGATAAGAGTACCCAGAATAAAATGAATAACTTTTTAAAGACAAAATCCTCTGTTATAATATTGCTAAAATTATTCAGAGTAATATTGTGGATTAAAGCC
ACAATAGAATAACATGTTAGACCATATTCAGTAGAAAAAGATGAACAATTAACTGATAAATTTGTGCACATGGCAAATTAGTTAATGGGAACCATAGGAGAATTTATTTCTAGATGTAAA
TAATTATTTTAAGTTTGCCCTATGGTGGCCCCACACATGAGACAAACCCCCAAGATGTGACTTTTGAGAATGAGACTTGGATAAAAAACATGTAGAAATGCAAGCCCTGAAGCTCAACTC
CCTATTGCTATCACAGGGGTTATAATTGCATAAAATTTAGCTATAGAAAGTTGCTGTCATCTCTTGTGGGCTGTAATCATCGTCTAGGCTTAAGAGTAATATTGCAAAACCTGTCATGCC
CACACAAATCTCTCCCTGGCATTGTTGTCTTTGCAGATGTCAGTGAAAGAGAACCAGCAGCTCCCATGAGTTTGGATAGCCTTATTTTCTATAGCCTCCCCACTATTAGCTTTGAAGGGA
GCAAAGTTTAAGAACCAAATATAAAGTTTCTCATCTTTATAGATGAGAAAAATTTTAAATAAAGTCCAAGATAATTAAATTTTTAAGGATCATTTTTAGCTCTTTAATAGCAATAAAACT
CAATATGACATAATATGGCACTTCCAAAATCTGAATAATATATAATTGCAATGACATACTTCTTTTCAGAGATTTACTGAAAAGAAATTTGTTGACACTACATAACGTGATGAGTGGTTT
ATACTGATTGTTTCAGTTGGTCTTCCCACCAACTCCATGAAAGTGGATTTTATTATCCTCATCATGCAGATGAGAATATTGAGACTTATAGCGGTATGCCTGGCCCAAGTACTCAGAGTT
------Intron 12
GCCTGGCTCCAAGATTTATAATCTTAAATGATGGGACTACCATCCTTACTCTCTCCATTTTTCTATACGTGAGTAATGTTTTTTCTGTTTTTTTTTTTTCTTTTTCCATTCAAACTCAG
Exon 13------(133 bp)
r ala leu val glu leu val lys his lys pro lys ala thr lys glu gln leu lys ala val met asp asp phe ala ala phe val glu
T GCA CTT GTT GAG CTC GTG AAA CAC AAG CCC AAG GCA ACA AAA GAG CAA CTG AAA GCT GTT ATG GAT GAT TTC GCA GCT TTT GTA GAG
------Exon 13
Intron 13------(614 bp)
lys cys cys lys ala asp asp lys glu thr cys phe ala glu glu
AAG TGC TGC AAG GCT GAC GAT AAG GAG ACC TGC TTT GCC GAG GAG
GTACTACAGTTCTCTTCATTTTAATATGTCCAGTATTCATTTTTGCATGTTTGGTTAGGC
TAGGGCTTAGGGATTTATATATCAAAGGAGGCTTTGTACATGTGGGACAGGGATCTTATTTTACAAACAATTGTCTTACAAAATGAATAAAACAGCACTTTGTTTTTATCTCCTGCTCTA
TTGTGCCATACTGTTGAATGTTTATAATGCATGTTCTGTTTCCAAATTTGTGATGCTTATGAATATTAATAGGAATATTTGTAAGGCCTGAAATATTTTGATCATGAAATCAAAACATTA
ATTTATTTAAACATTTACTTGAAATGTGGTGGTTTGTGATTTAGTTGATTTTATAGGCTAGTGGGAGAATTTACATTCAAATGTCTAAATCACTTAAAATTTCCCTTTATGGCCTGACAG
TAACTTTTTTTTATTCATTTGGGGACAACTATGTCCGTGAGCTTCCATCCAGAGATTATAGTAGTAAATTGTAATTAAAGGATATGATGCACGTGAAATCACTTTGCAATCATCAATAGC

FIG. IB-12

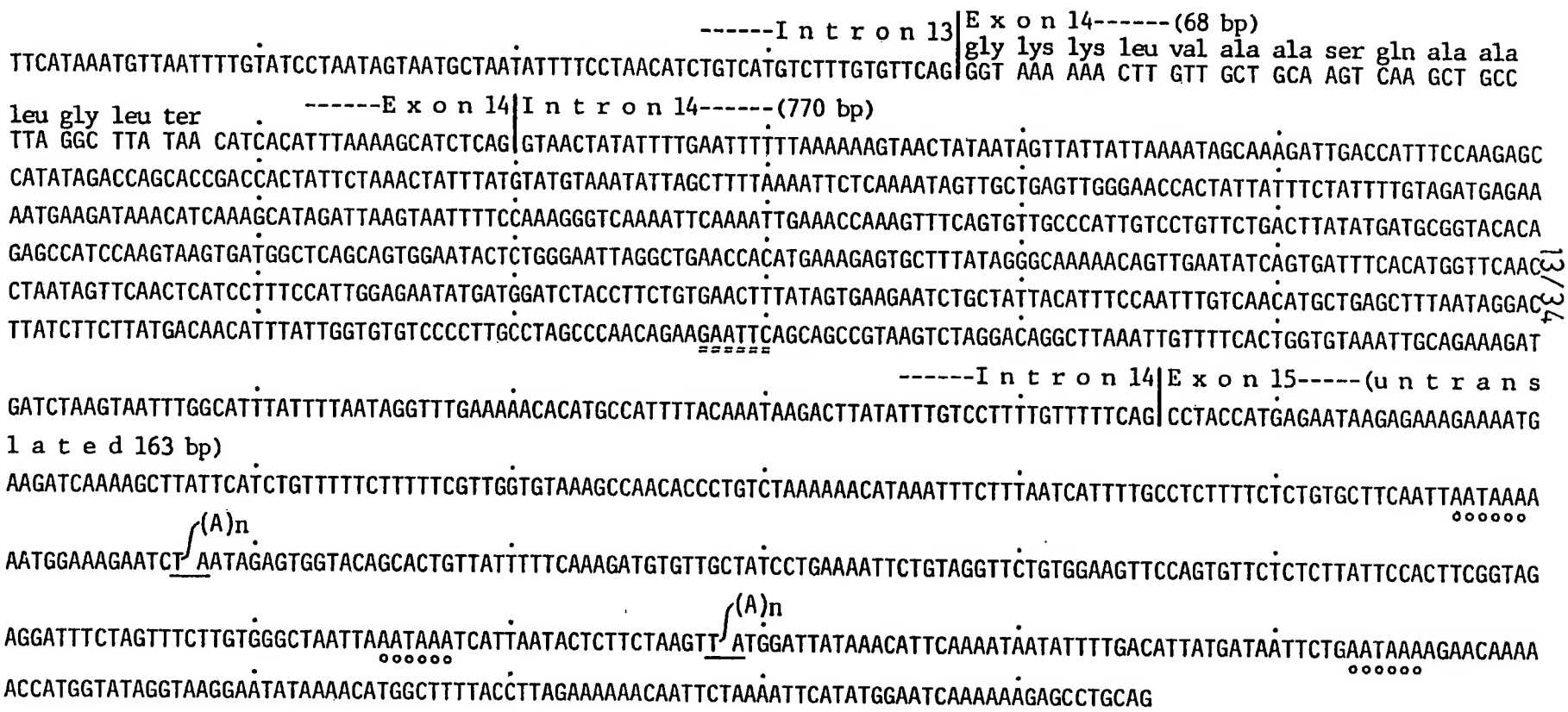
------Intron 13|Exon 14------(68 bp)
gly lys lys leu val ala ala ser gln ala ala
TTCATAAATGTTAATTTTGTATCCTAATAGTAATGCTAATATTTTCCTAACATCTGTCATGTCTTTGTGTTCAG|GGT AAA AAA CTT GTT GCT GCA AGT CAA GCT GCC
leu gly leu ter ------Exon 14|Intron 14------(770 bp)
TTA GGC TTA TAA CATCACATTTAAAAGCATCTCAG|GTAACTATATTTTGAATTTTTTAAAAAAGTAACTATAATAGTTATTATTAAAATAGCAAAGATTGACCATTTCCAAGAGC
CATATAGACCAGCACCGACCACTATTCTAAACTATTTATGTATGTAAATATTAGCTTTTAAAATTCTCAAAATAGTTGCTGAGTTGGGAACCACTATTATTTCTATTTTGTAGATGAGAA
AATGAAGATAAACATCAAAGCATAGATTAAGTAATTTTCCAAAGGGTCAAAATTCAAAATTGAAACCAAAGTTTCAGTGTTGCCCATTGTCCTGTTCTGACTTATATGATGCGGTACACA
GAGCCATCCAAGTAAGTGATGGCTCAGCAGTGGAATACTCTGGGAATTAGGCTGAACCACATGAAAGAGTGCTTTATAGGGCAAAAACAGTTGAATATCAGTGATTTCACATGGTTCAAC
CTAATAGTTCAACTCATCCTTTCCATTGGAGAATATGATGGATCTACCTTCTGTGAACTTTATAGTGAAGAATCTGCTATTACATTTCCAATTTGTCAACATGCTGAGCTTTAATAGGAC
TTATCTTCTTATGACAACATTTATTGGTGTGTCCCCTTGCCTAGCCCAACAGAAGAATTCAGCAGCCGTAAGTCTAGGACAGGCTTAAATTGTTTTCACTGGTGTAAATTGCAGAAAGAT
------Intron 14|Exon 15-----(untrans
GATCTAAGTAATTTGGCATTTATTTTAATAGGTTTGAAAAACACATGCCATTTTACAAATAAGACTTATATTTGTCCTTTTGTTTTTCAG|CCTACCATGAGAATAAGAGAAAGAAAATG
lated 163 bp)
AAGATCAAAAGCTTATTCATCTGTTTTTCTTTTTCGTTGGTGTAAAGCCAACACCCTGTCTAAAAAACATAAATTTCTTTAATCATTTTGCCTCTTTTCTCTGTGCTTCAATTAATAAAA
AATGGAAAGAATCT(A)nAATAGAGTGGTACAGCACTGTTATTTTTCAAAGATGTGTTGCTATCCTGAAAATTCTGTAGGTTCTGTGGAAGTTCCAGTGTTCTCTCTTATTCCACTTCGGTAG
AGGATTTCTAGTTTCTTGTGGGCTAATTAAATAAATCATTAATACTCTTCTAAGTT(A)nATGGATTATAAACATTCAAAATAATATTTTGACATTATGATAATTCTGAATAAAAGAACAAAA
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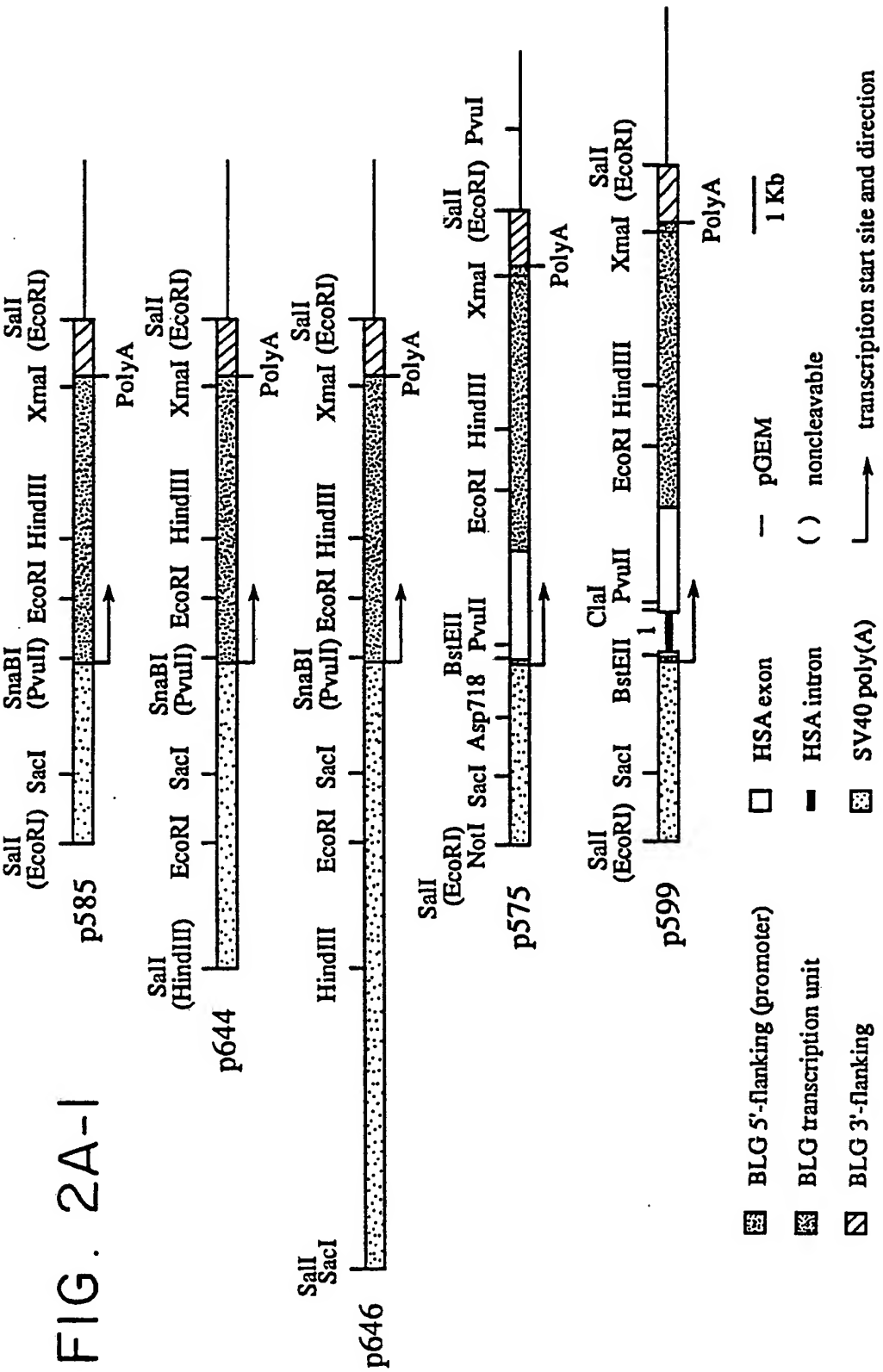
FIG. 2A-1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SacI
SnaBI (PvuII)
EcoRI
HindIII
XmaI
SalI (EcoRI)
PolyA
p644
SalI (HindIII)
EcoRI
SacI
SnaBI (PvuII)
EcoRI
HindIII
XmaI
SalI (EcoRI)
PolyA
p646
SalI
SacI
HindIII
EcoRI
SacI
SnaBI (PvuII)
EcoRI
HindIII
XmaI
SalI (EcoRI)
PolyA
p575
SalI (EcoRI)
NotI
SacI
Asp718
BstEII
PvuII
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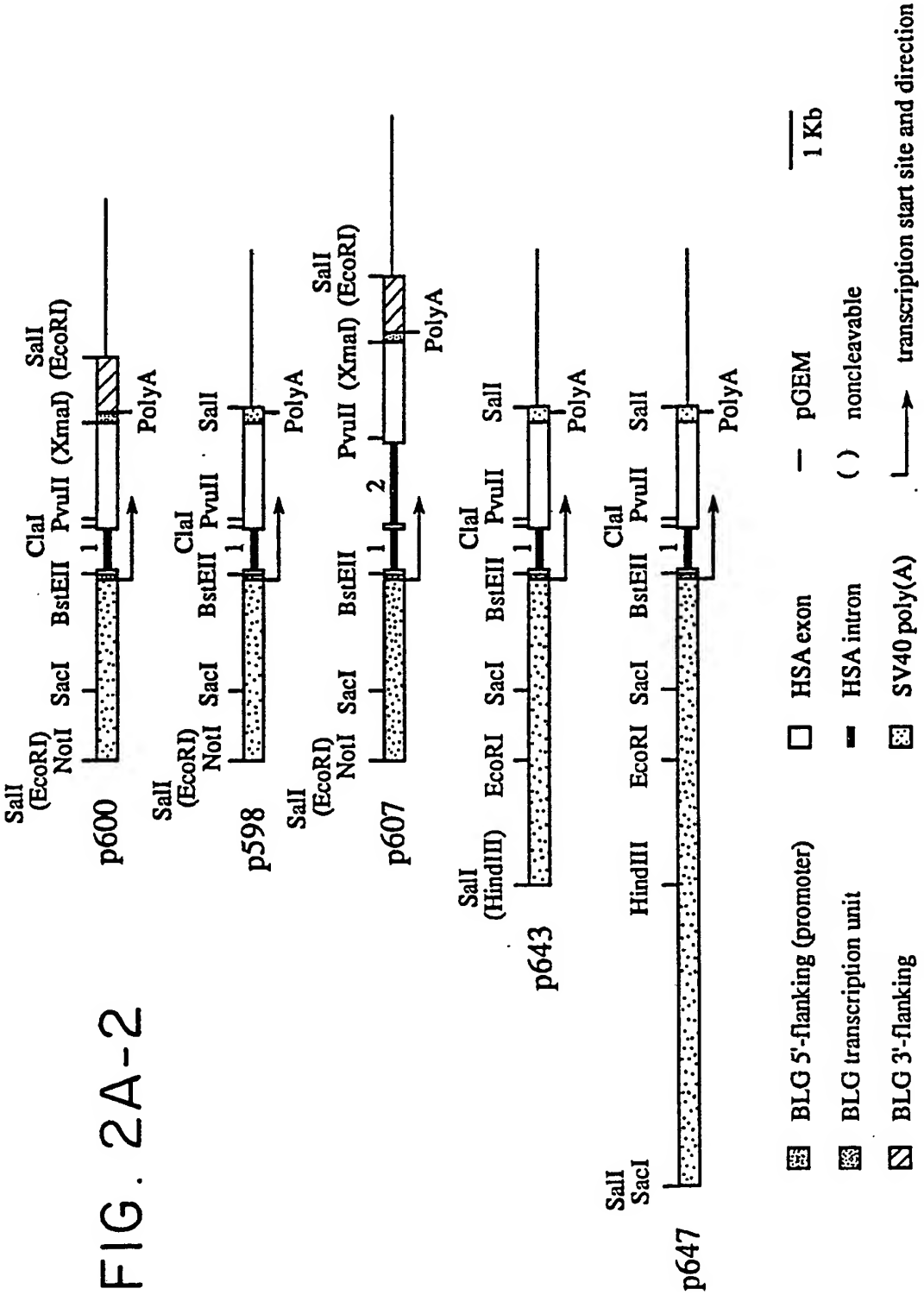
HindIII
XmaI
SalI (EcoRI)
PvuI
PolyA
p599
SalI (EcoRI)
SacI
BstEII
ClaI
PvuII
EcoRI
HindIII
XmaI
SalI (EcoRI)
1
PolyA
BLG 5'-flanking (promoter)
HSA exon
pGEM
1 Kb
BLG transcription unit
HSA intron
( ) noncleavable
BLG 3'-flanking
SV40 poly(A)
transcription start site and direction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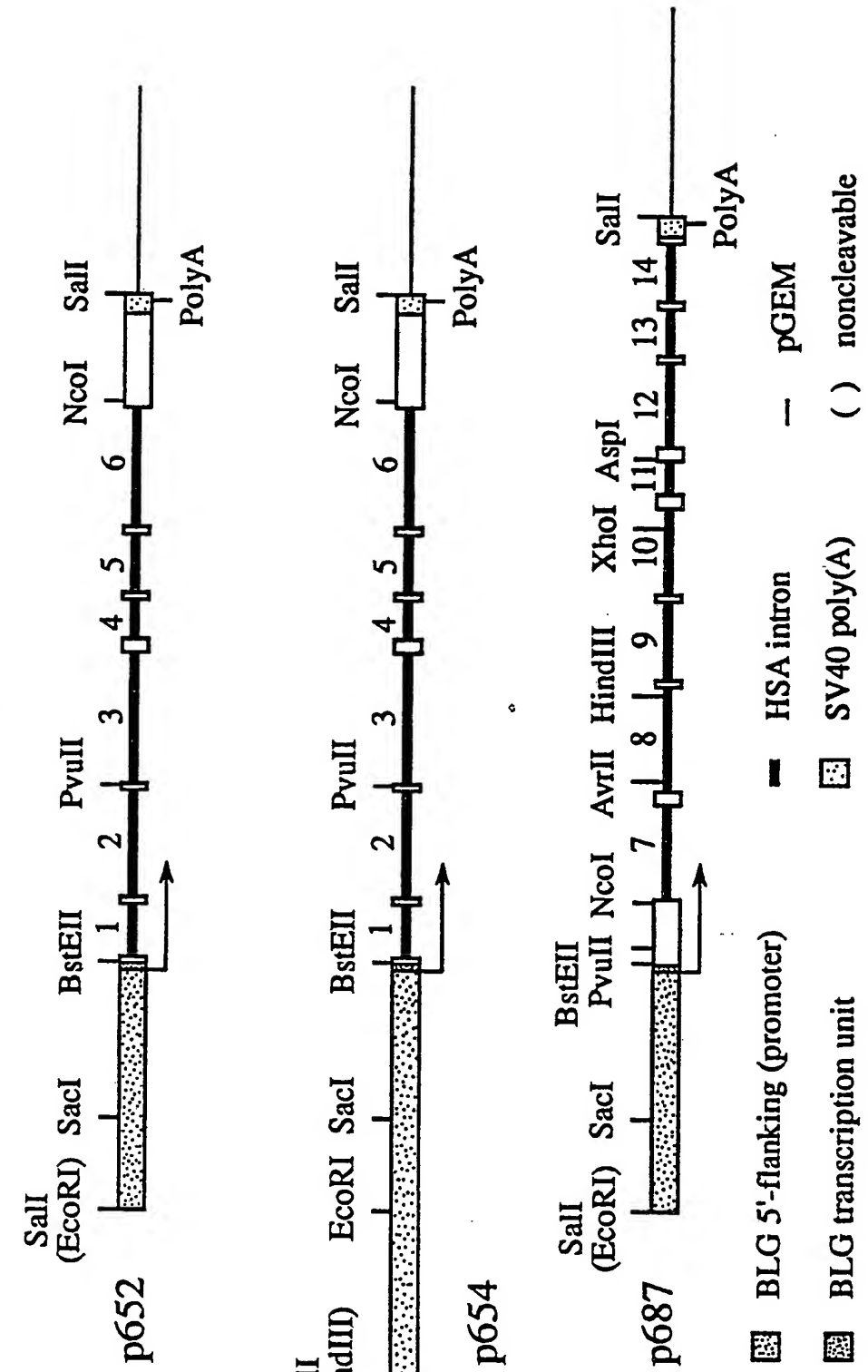
NotI
SacI
BstEII
ClaI
PvuII (XmaI)
SalI (EcoRI)
PolyA
p600
p598
p607
p643
p647
SalI (HindIII)
EcoRI
HindIII
1
2
BLG 5'-flanking (promoter)
BLG transcription unit
BLG 3'-flanking
HSA exon
HSA intron
SV40 poly(A)
pGEM
noncleavable
1 Kb
transcription start site and direction

FIG. 2A-2

FIG. 2B-1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PvuII
NcoI
SalI
PolyA
p652
SalI (HindIII)
EcoRI
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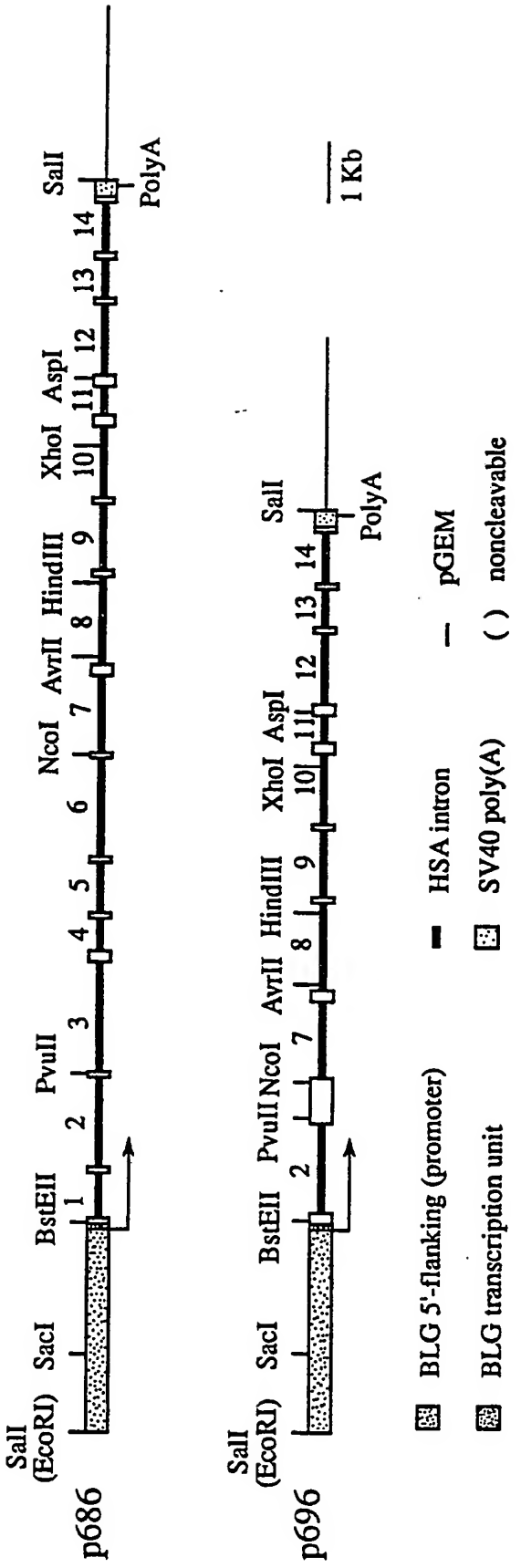
PvuII
NcoI
SalI
PolyA
p654
SalI (EcoRI)
SacI
BstEII
PvuII
NcoI
AvrII
HindIII
XhoI
AspI
SalI
PolyA
p687
BLG 5'-flanking (promoter)
HSA intron
pGEM
BLG transcription unit
SV40 poly(A)
( ) noncleavable
HSA exon

FIG. 2B-2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SacI
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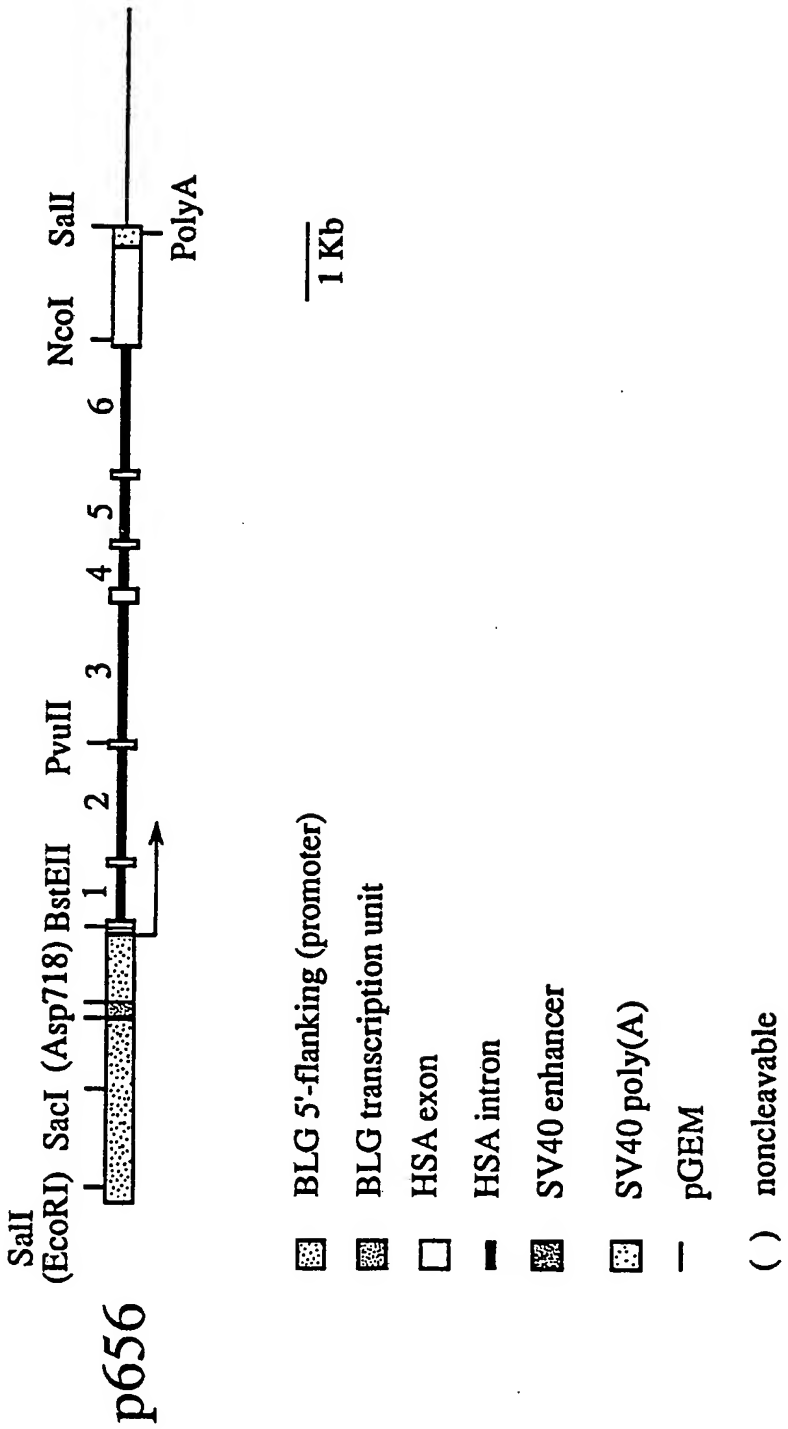
PvuII
NcoI
AvrII
HindIII
XhoI
AspI
SalI
PolyA
1 Kb
p696
BLG 5'-flanking (promoter)
BLG transcription unit
HSA exon
HSA intron
pGEM
SV40 poly(A)
( ) noncleavable

FIG. 2C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SacI
(Asp718)
BstEII
PvuII
NcoI
SalI
PolyA
1
2
3
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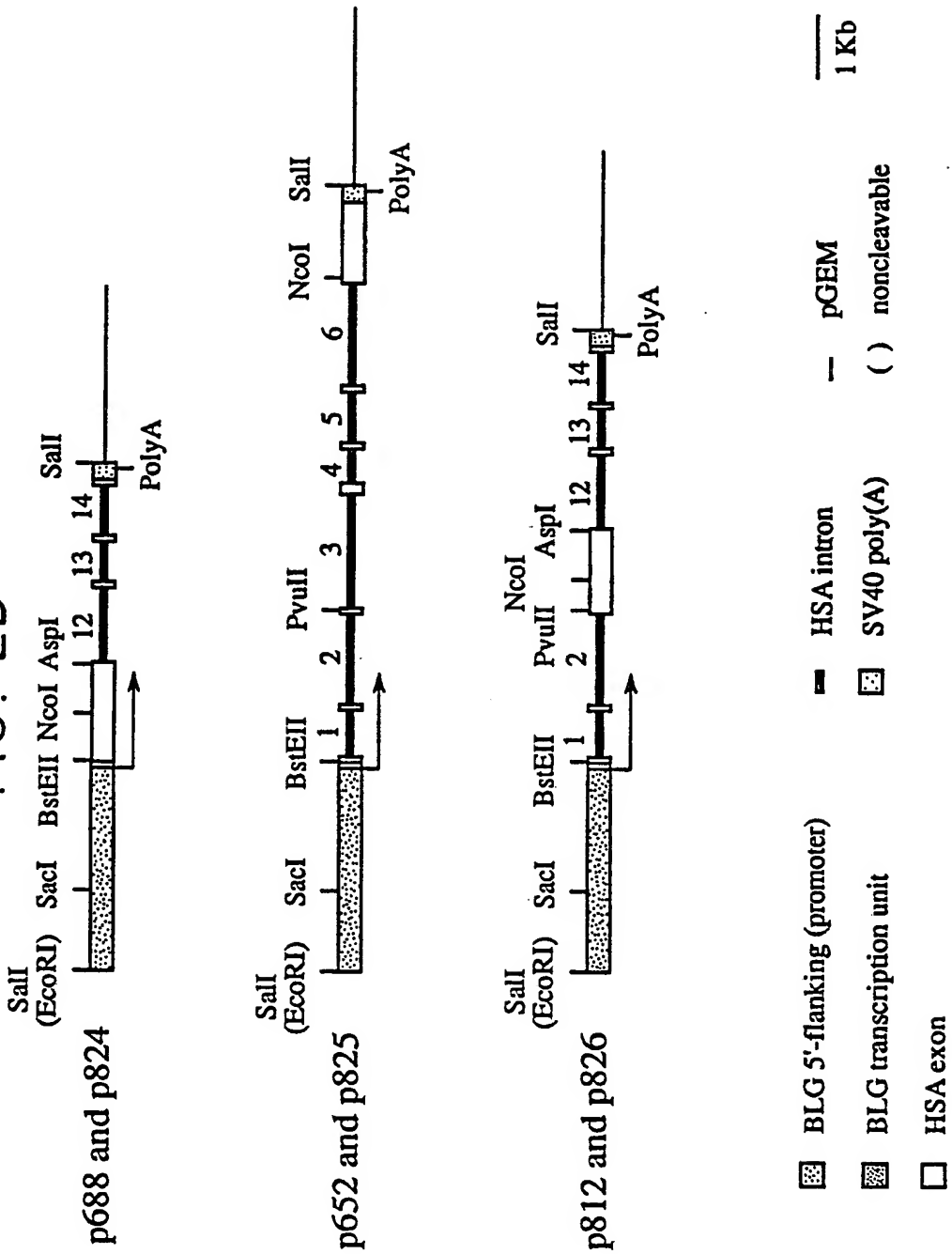
5
6
1 Kb
BLG 5'-flanking (promoter)
BLG transcription unit
HSA exon
HSA intron
SV40 enhancer
SV40 poly(A)
pGEM
( ) noncleavable

FIG. 2D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NcoI
AspI
SalI
PolyA
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PvuII
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p652 and p825
p812 and p826
1 Kb
pGEM
HSA intron
SV40 poly(A)
noncleavable
BLG 5'-flanking (promoter)
BLG transcription unit
HSA exon

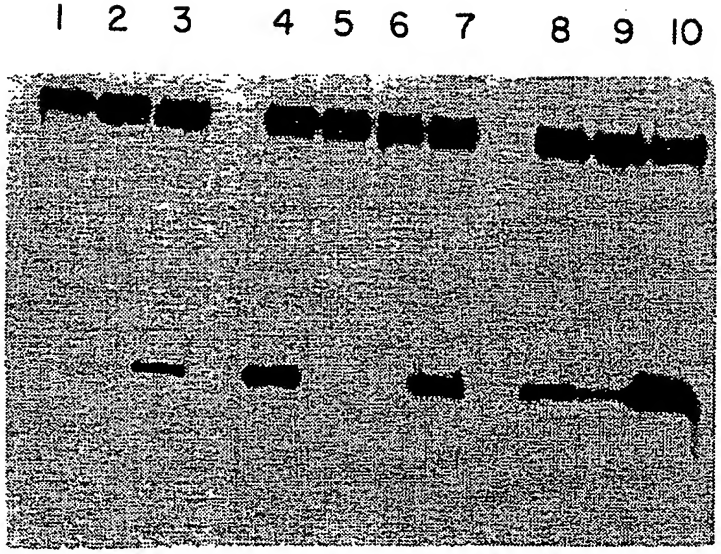


FIG. 3A

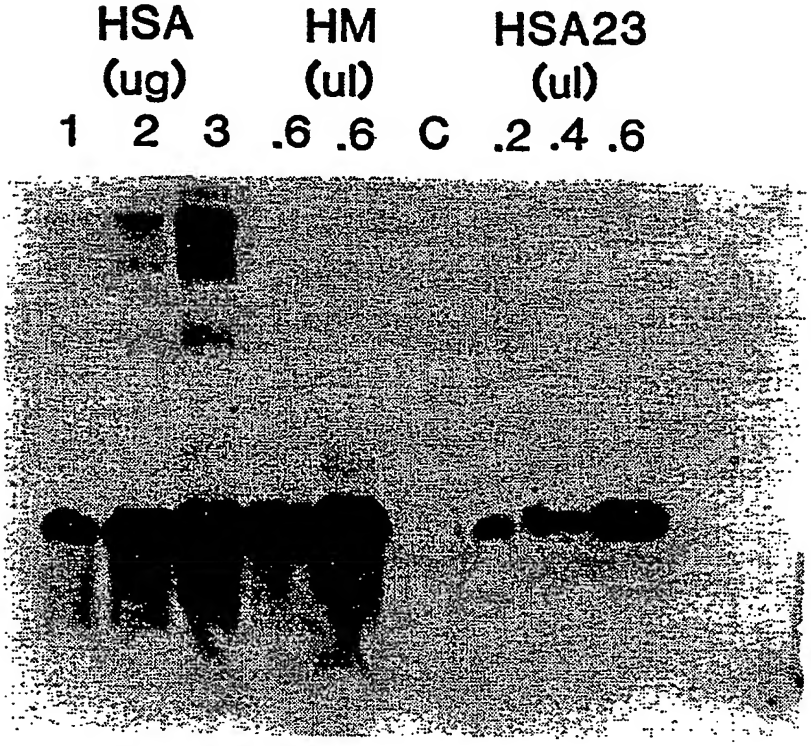


FIG. 6C

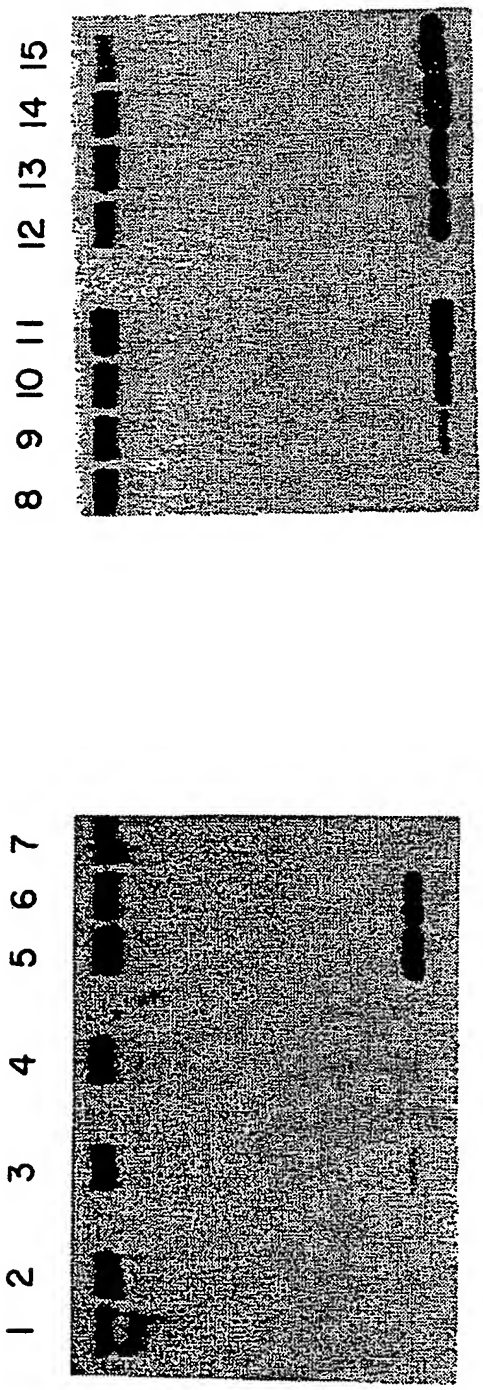


FIG. 3B

FIG. 4

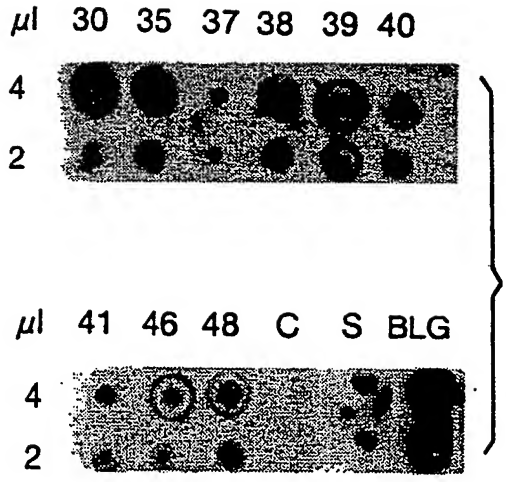


FIG. 5A

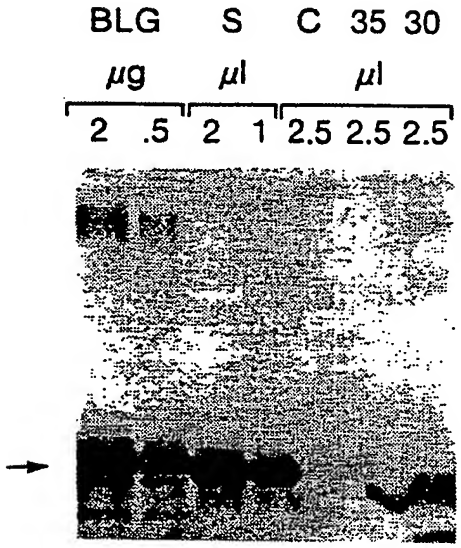


FIG. 5B

HSA (ng)	500	250	125	60	30	15	7	3	1	.6	.3
SAMP.	C	HM	11	12	15	17	24	25	9	16	14
SAMP.	16	18	20	22	23	26	27	28	29	S	27

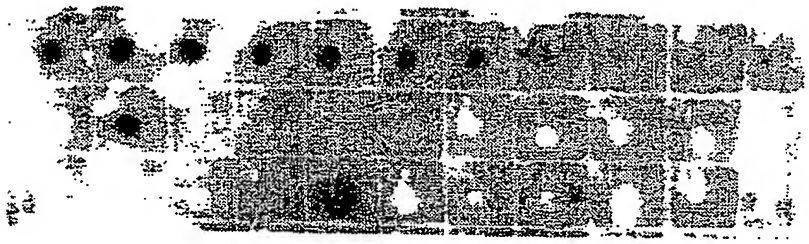


FIG. 6A

HSA (ng)	1000	500	250	125	60	30	15	7	3	1
SAMP. (ul)	22 .6	.4	.2	.1	23 .6	.4	.2	.1	.05	.02
SAMP. (ul)	23 .01	.005	.002	C .2	HM .2					

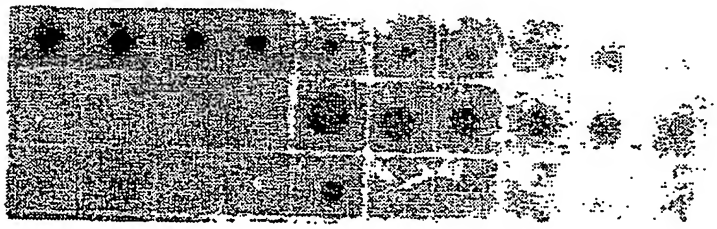


FIG. 6B

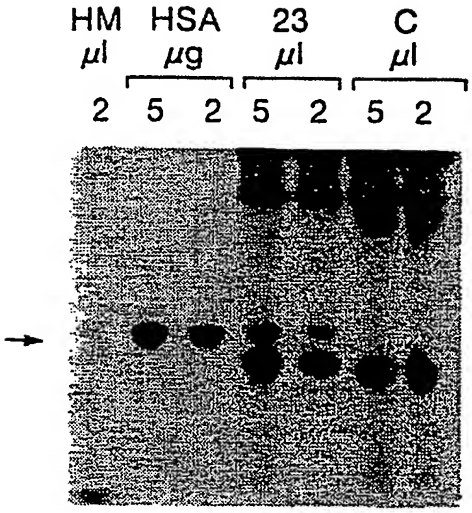


FIG. 7A

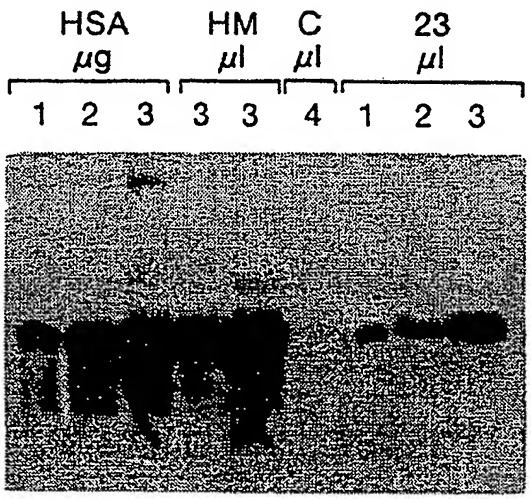


FIG. 7B

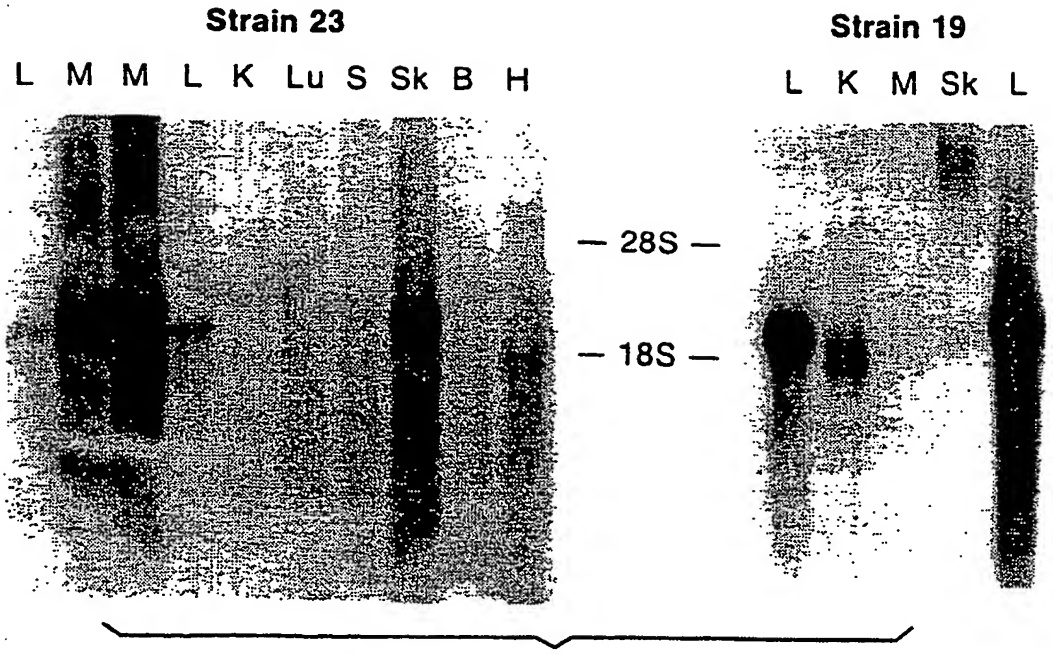


FIG. 8

FIG. 9A

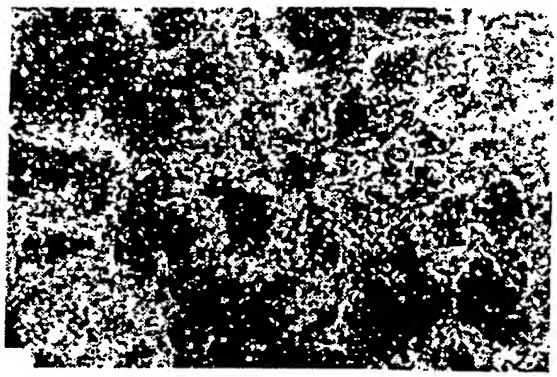


FIG. 9B

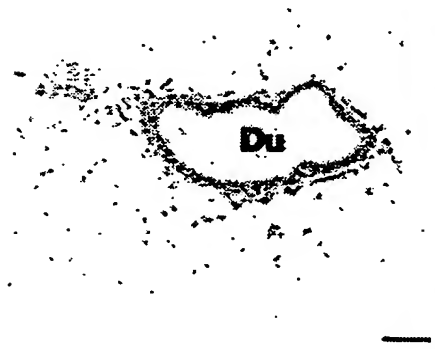


FIG. 9C

FIG. 9D

FIG. 9E

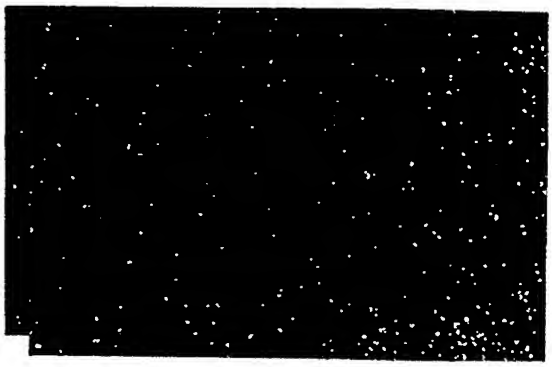
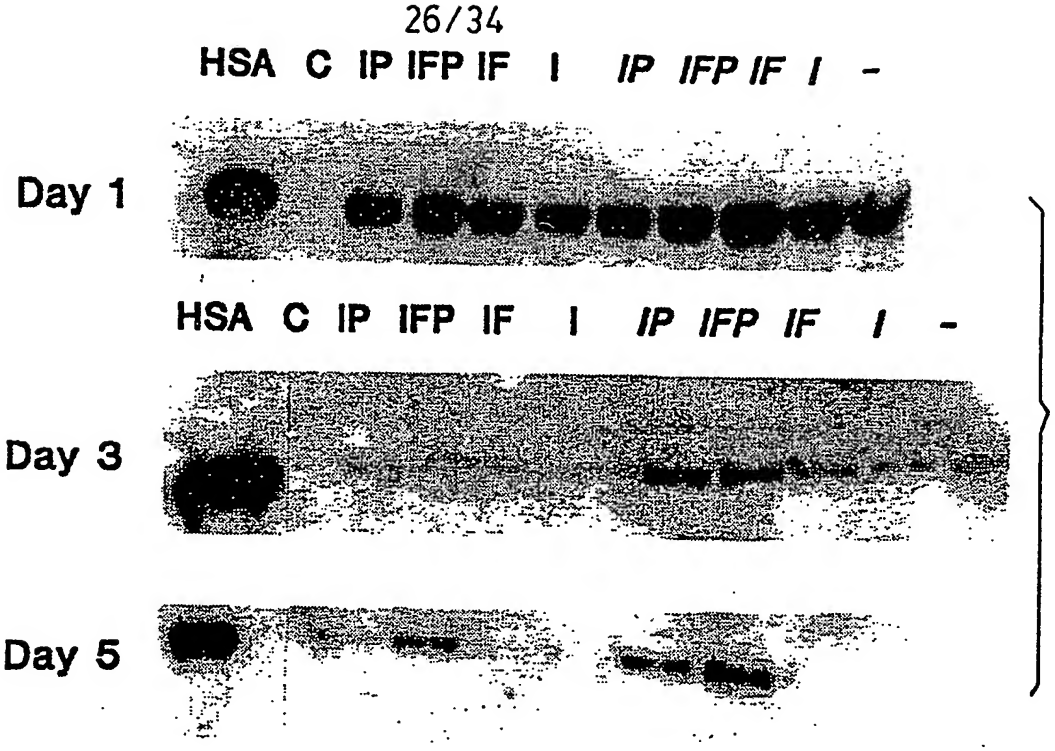


FIG. 9F



I & F at 0.1 ug/ml, P at 1.0 ug/ml
I, F & P at 5 ug/ml

FIG. 10

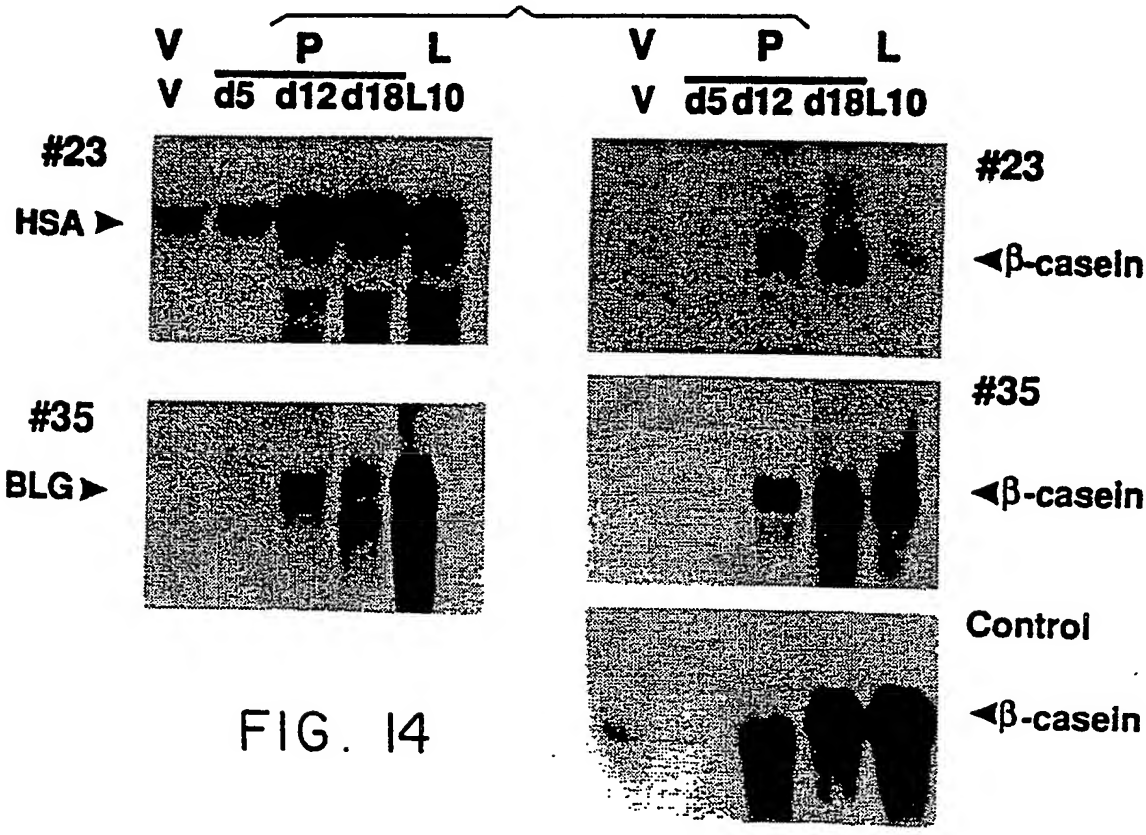


FIG. 14

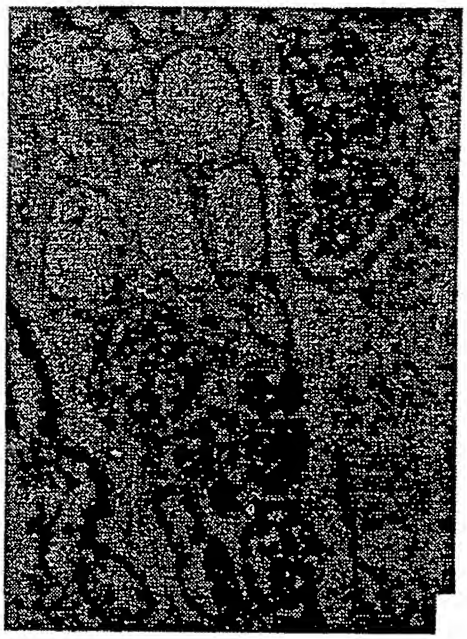


FIG. 11B

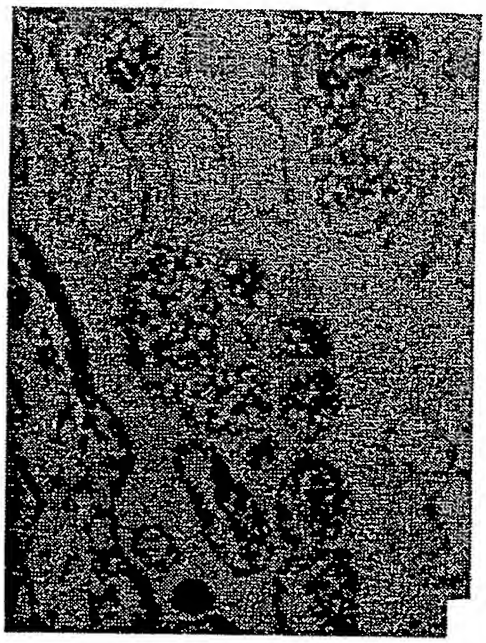


FIG. 11D

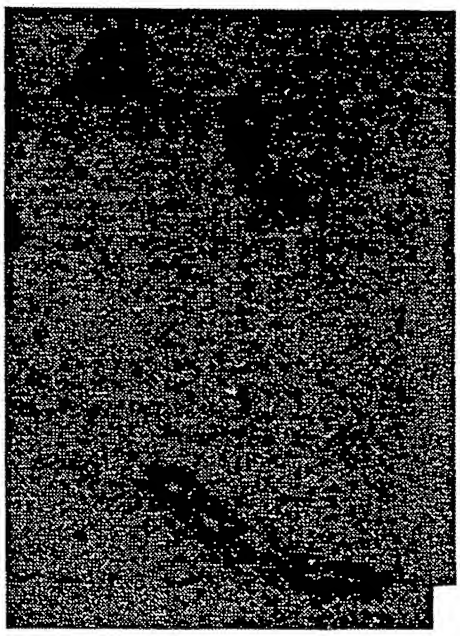


FIG. 11A

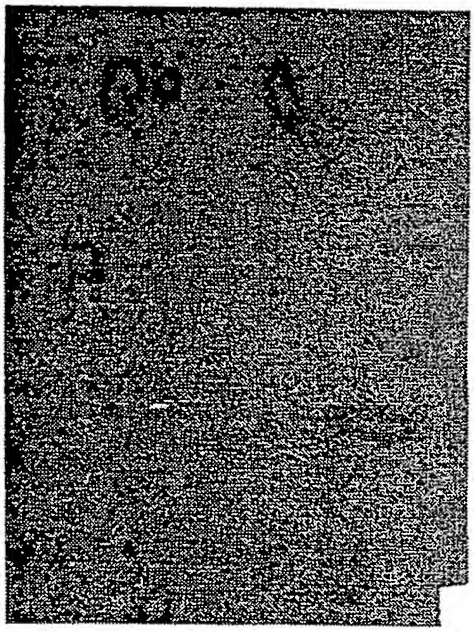


FIG. 11C

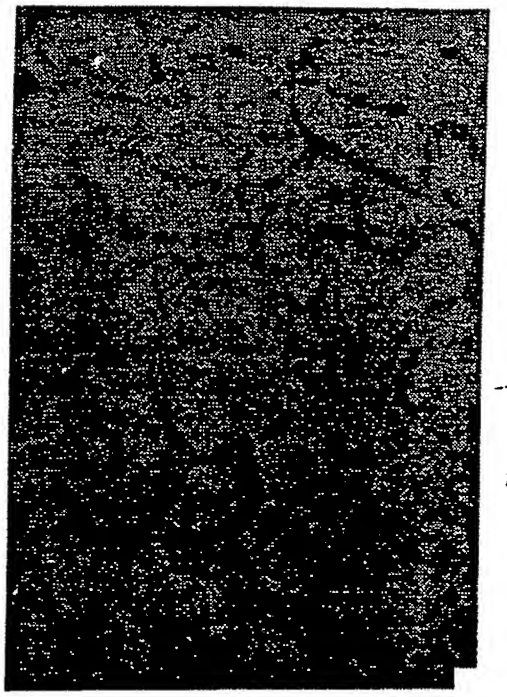


FIG. 12B

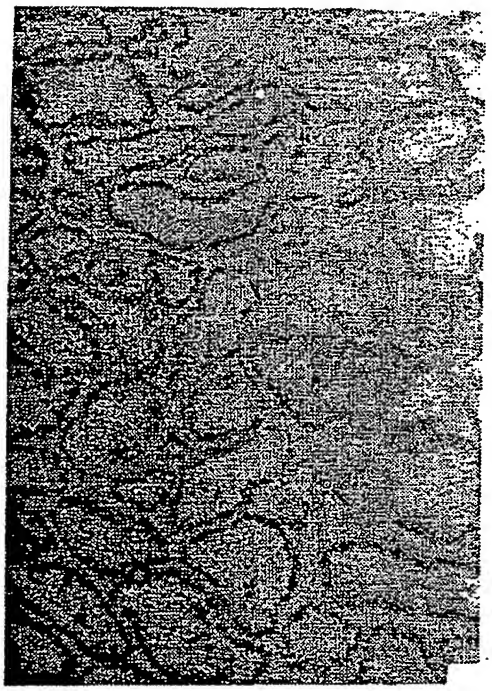


FIG. 12D

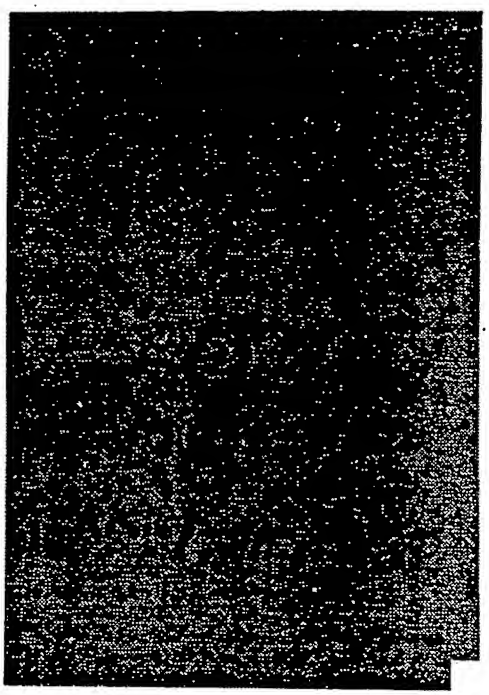


FIG. 12A

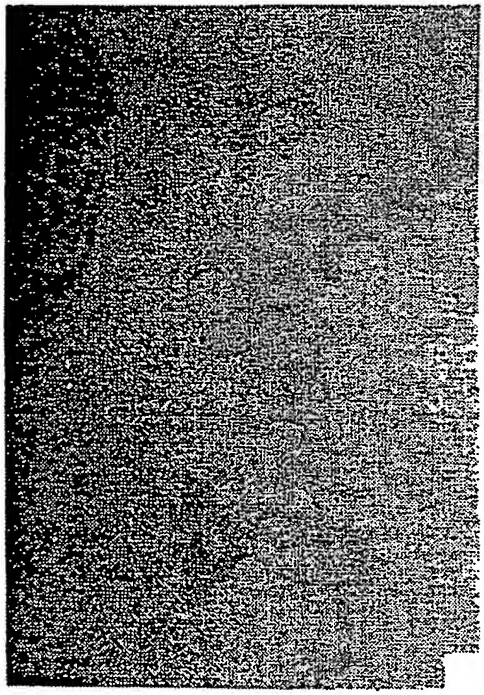


FIG. 12C

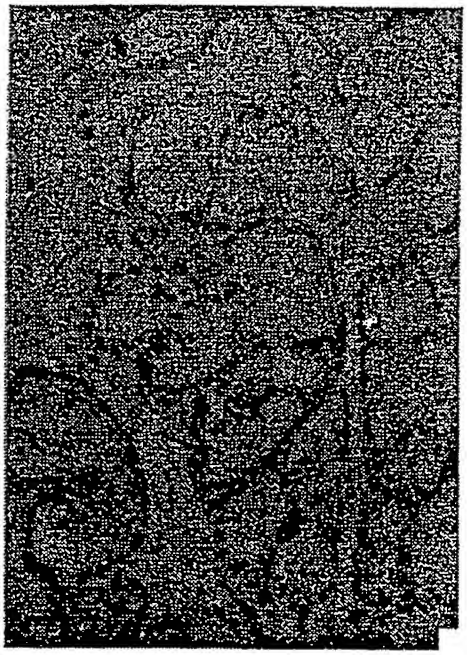


FIG. 13B

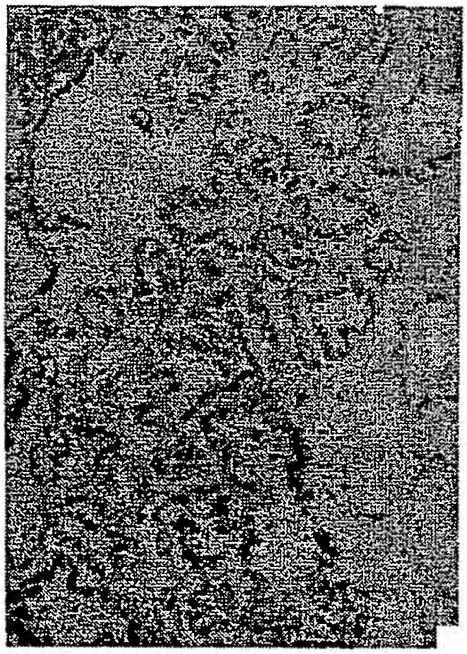


FIG. 13D

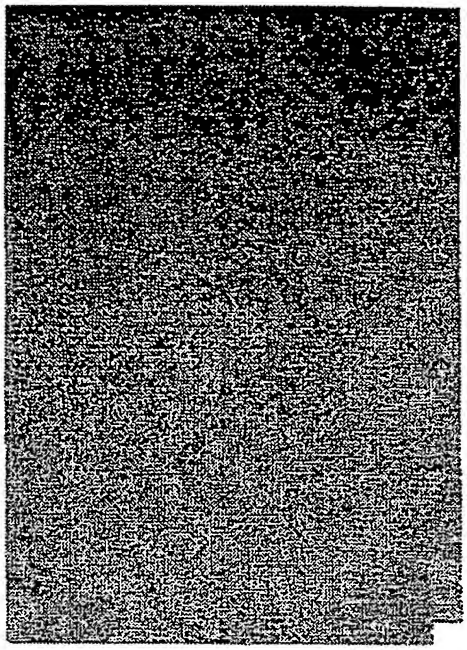


FIG. 13A

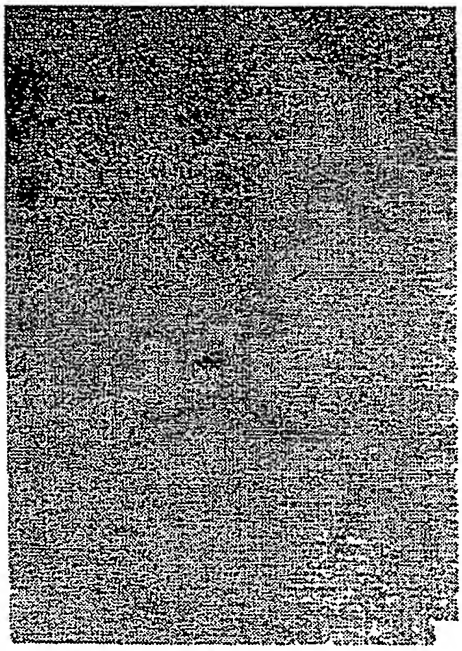


FIG. 13C

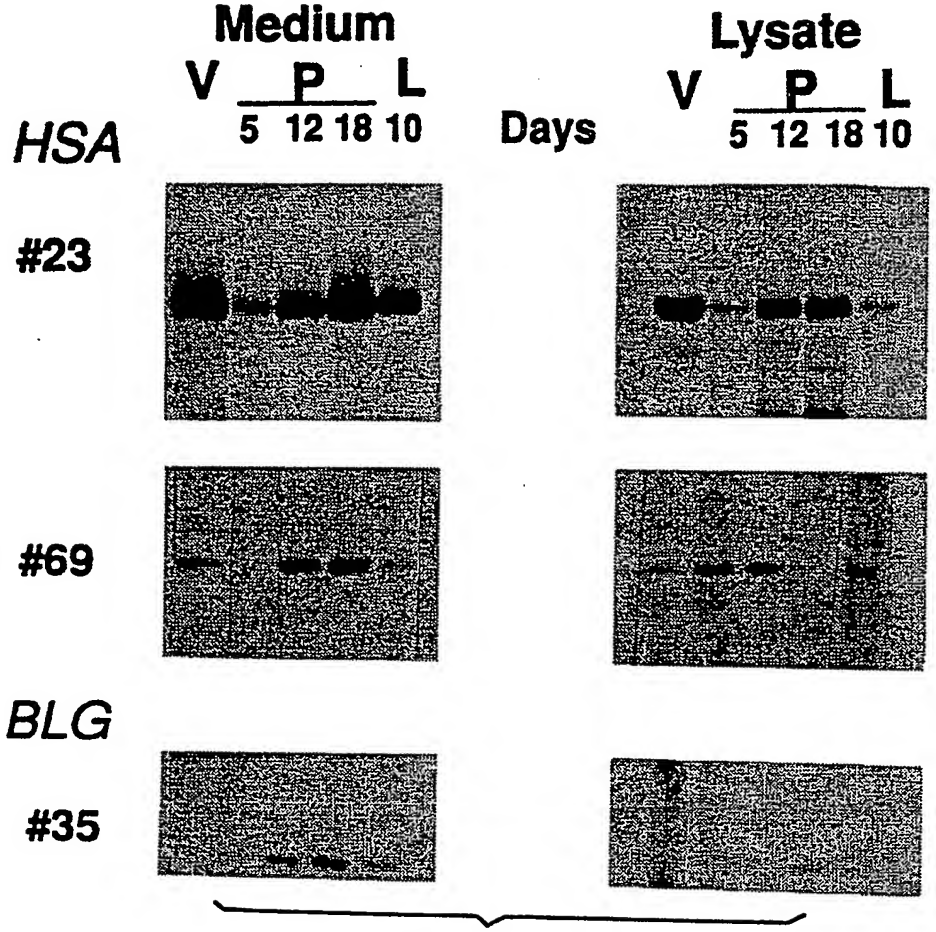

Medium
V P L
5 12 18 10
Days
Lysate
V P L
5 12 18 10
HSA
#23
#69
BLG
#35

FIG. 15

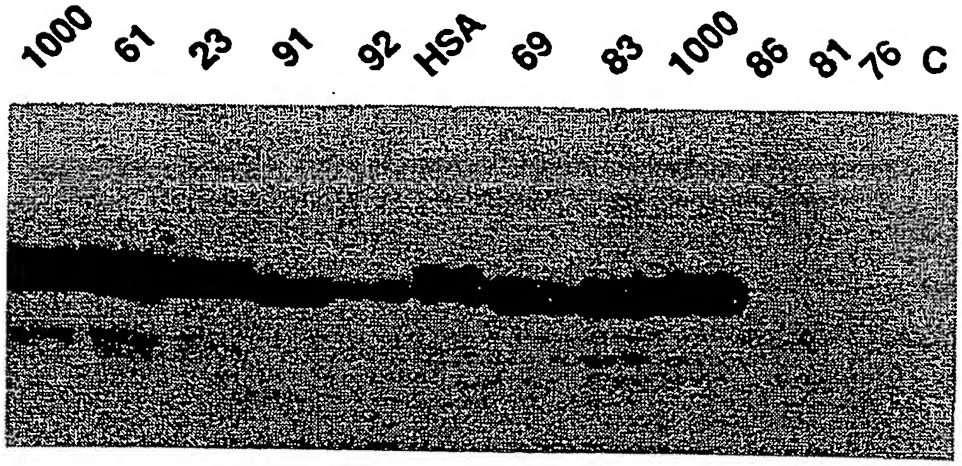

1000
61
23
91
92
HSA
69
83
1000
86
81
76
C

FIG. 17

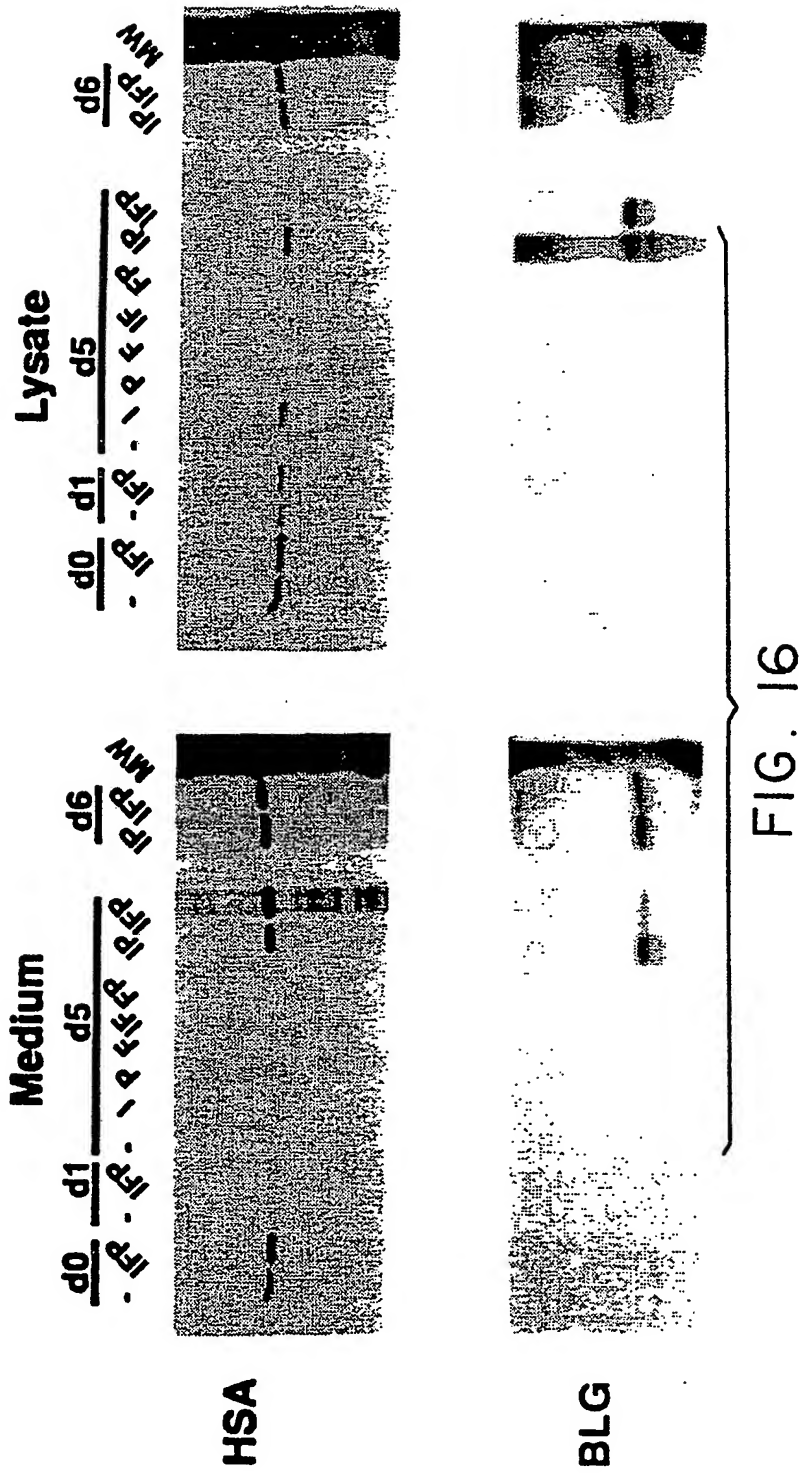


FIG. 16

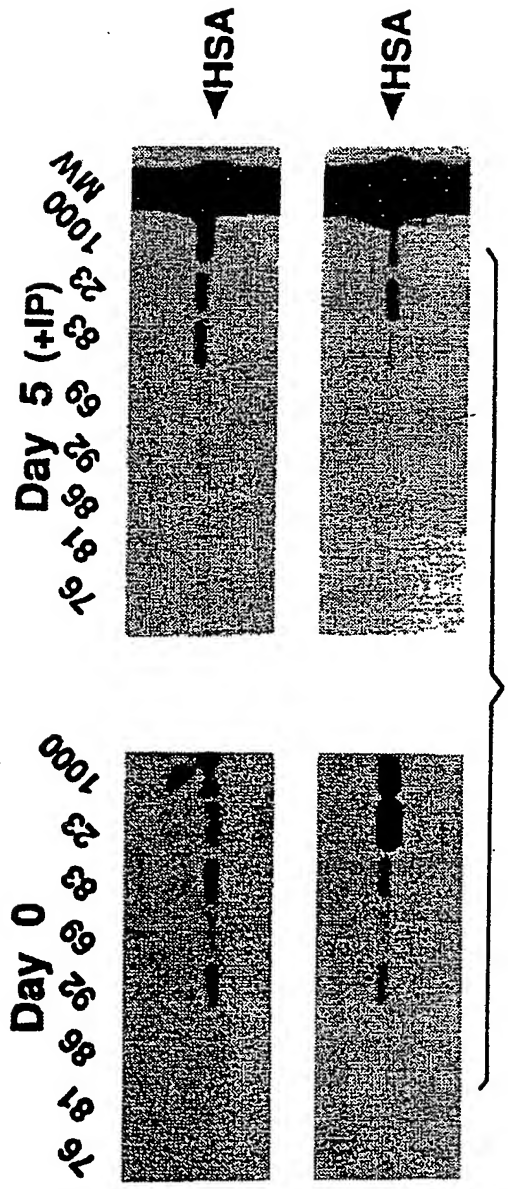


FIG. 18A

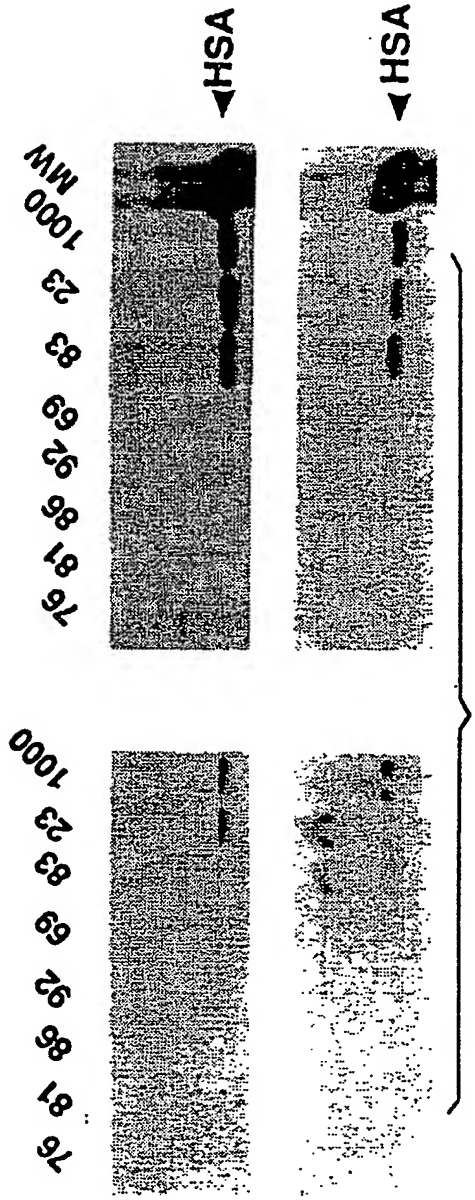
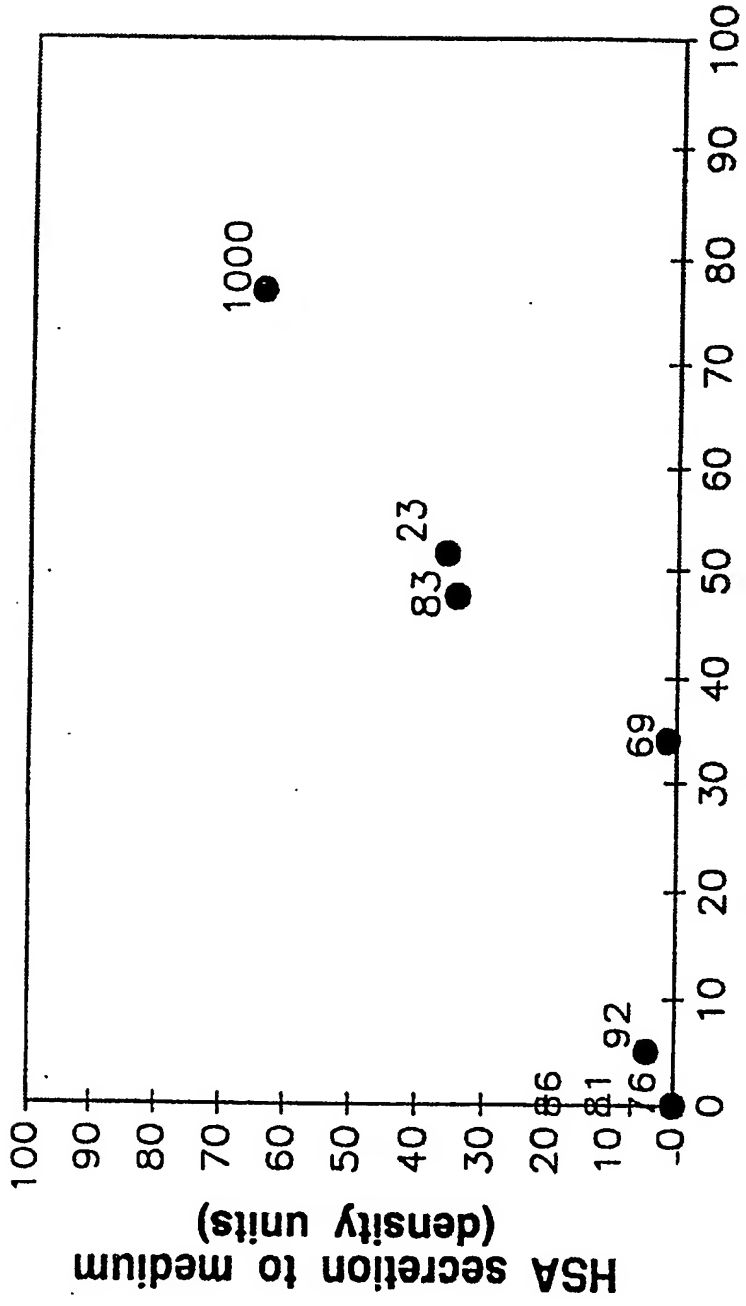


FIG. 18B



Explants were incubated for 5 days in the presence of insulin and prolactin

*Equal amounts of TCA precipitable proteins /lane

FIG. 19

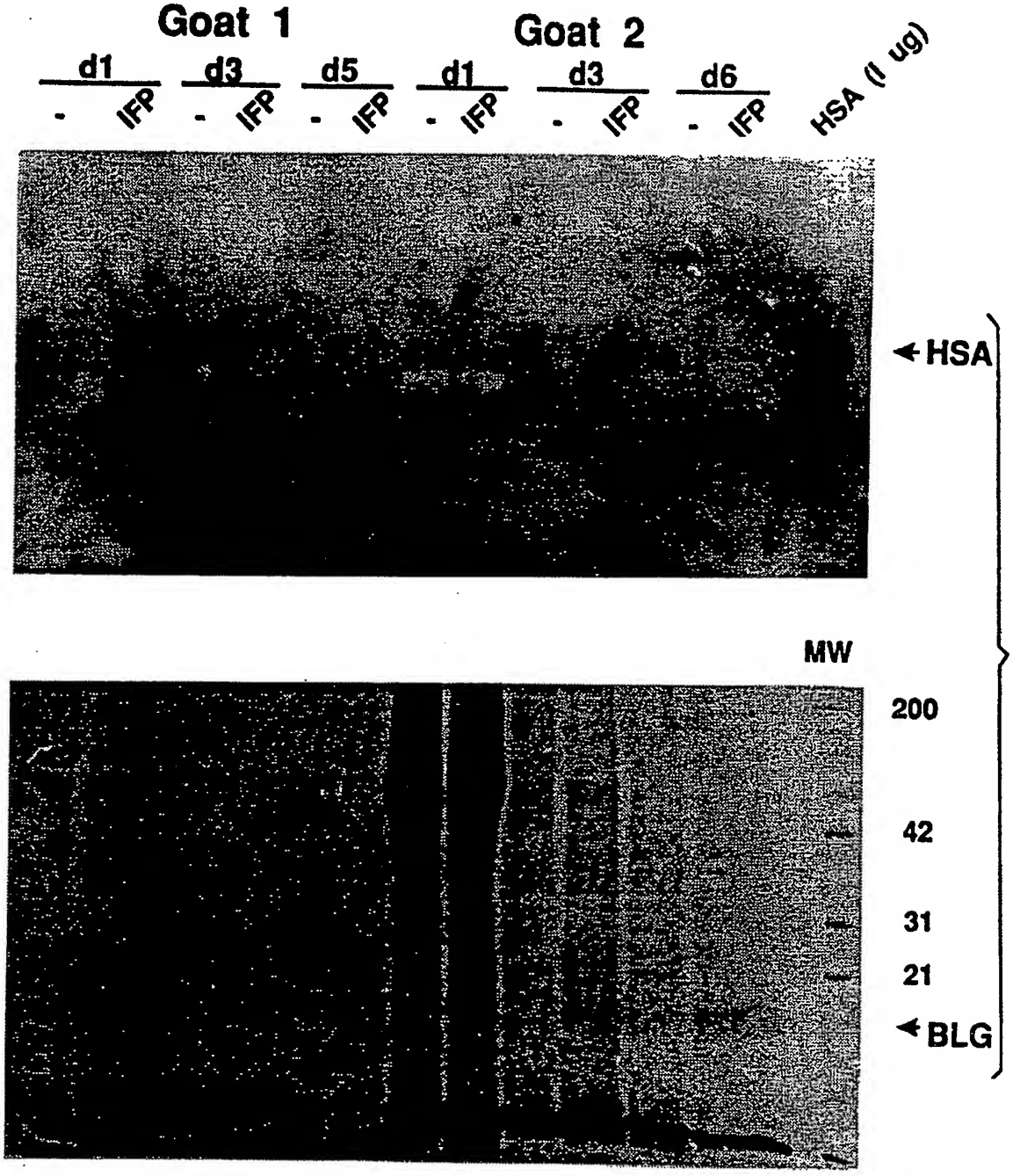


FIG. 20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/06300

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/90, 15/67, 15/14; C07H 21/04; A01K 67/02; G01N 33/04

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

GENES III

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proc. Natl. Acad. Sci. USA, Vol. 85, issued February 1988, Brinster et al., "Introns increase transcriptional efficiency in transgenic mice, pages 836-840, see the entire document.	1-14
Y	US, A, 4,873,316 (Meade et al.) 10 October 1989, see the entire document.	1-14
X	Proc. Natl. Acad. Sci. USA, Vol. 88, issued July 1991, Watkins et al., "A donor splice mutation and a single-base deletion produce two carboxyl-terminal variants of human serum albumin", pages 5959-5963, see the entire document.	1,6
A	Journal of Biological Chemistry, Volume 261, No. 15, issued 25 May 1986, Minghetti et al., "Molecular Structure of the Human Albumin Gene Is Revealed by Nucleotide Sequence within q11-22 of Chromosome 4", pages 6747-6757, see the entire document.	1-14

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 September 1992	Date of mailing of the international search report 07 OCT 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer JACQUELINE STONE Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/06300

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.6, 172.2; 800/002, DIG 1; 536/27; 424/009; 119/174; 935/10, 33, 36, 53, 63, 93

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